Vol. 49 July 1960 No. 7

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

CONTENTS OF THIS ISSUE

The Avidity of Salicylic, Gentisic, and Salicyluric Acids for Heavy Metal Cations 411
Joseph Pecci and *William O. Foye*

Relationship of Chemical Structure to Central Nervous System Effects of Tranquilizing and Anticonvulsant Drugs 415
T. C. Barnes

Complexing Tendencies of Saccharin in Aqueous Solutions 417
J. R. Marvel and *A. P. Lemberger*

An Investigation of Solvent Effect Upon Saccharin Complexes With N,N-Dimethylacetamide 420
J. R. Marvel and *A. P. Lemberger*

The Preparation and Use of Radioiodinated Congo Red in Detecting Amyloidosis 422
Manuel Tubis, William H. Blahd, and *Robert A. Nordyke*

An Investigation of *Coprinus atramentarius* for the Presence of Disulfiram 426
J. K. Wier and *V. E. Tyler, Jr.*

Interaction of Preservatives With Macromolecules IV. Binding of Quaternary Ammonium Compounds by Nonionic Agents 430
Patrick P. DeLuca and *H. B. Kostenbauder*

Urinary Excretion Kinetics for Evaluation of Drug Absorption III. Method for Calculation of Absorption Rate and Application to Tetracycline Absorption in Humans 437
Eino Nelson

The Relationship of Metal Binding to the Biological Activities of Phenethylbiguanide 441
Eugene D. Weinberg, Robert Chernin, and *John H. Billman*

The Solubility of Benzoic Acid in Mixed Solvents 444
Marvin J. Chertkoff and *Alfred N. Martin*

The Photo-oxidation of Dihydroxyphenylalanine in the Presence of 8-Methoxypsoralen 447
Joseph Judis

Effects of Fatty Acids on Vitamin A Esters in Isopropanol Solutions 451
Albert J. Forlano and *Loyd E. Harris*

Preparation and Stability of Some Esters of Vitamin A 458
Albert J. Forlano and *Loyd E. Harris*

An Investigation of Particle-Medium Interactions in Suspensions 463
W. A. Haddad-Louis and *A. P. Lemberger*

Accelerated Color Loss of Certified Dyes in the Presence of Nonionic Surfactants 467
Morton W. Scott, Alex J. Goudie, and *Arthur J. Huetteman*

The Relationship Between Physiological Availability of Salicylates and Riboflavin and *In Vitro* Disintegration Time of Enteric Coated Tablets 473
A. B. Morrison and *J. A. Campbell*

Emulsification With Ultrasonic Waves I. Evaluation of the Minisonic and a Modified Analysis Procedure 478
Robert E. Singiser and *Harold M. Beal*

Emulsification With Ultrasonic Waves II. Evaluation of Three Ultrasonic Generators and a Colloid Mill 482
Robert E. Singiser and *Harold M. Beal*

Book Notices 488

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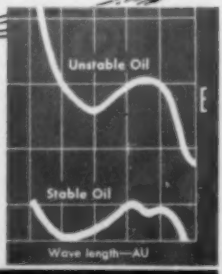

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Offices: Editorial, Advertising and Subscription Offices: 2215 Constitution Avenue, N.W., Washington 7, D.C. Publication Office: 20th and Northampton Streets, Easton, Pennsylvania.

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49 | *JULY 1960* | NUMBER 7

The Avidity of Salicylic, Gentisic, and Salicyluric Acids for Heavy Metal Cations*

By JOSEPH PECCI† and WILLIAM O. FOYE

Potentiometric titrations were carried out with the title compounds in the presence of various heavy metal cations. Evidence was provided by this method that Cu^{++}, Fe^{+++}, and Al^{+++} ions were capable of forming stable chelates with these acids. No evidence of chelation was found with Co^{++}, Ni^{++}, Zn^{++}, Mg^{++}, Ca^{++}, or Ag^{+} ions, although chelates of low stability would not be detected by this procedure. The avidities of these acids for metal ions were recorded as stability constants, and their magnitude indicates that the chelates should be capable of existence in the animal cell.

IT HAS BEEN suggested that salicylates may exert some, if not all, of their biological effects through their ability to chelate the ions of metals (1). The nonchelating *meta*- and *para*-hydroxybenzoic acids show none of the classical actions of the salicylates (2). It was therefore thought enlightening to examine salicylic acid and its major metabolites, salicyluric and gentisic acids, in regard to the heavy metal cations that they are capable of chelating, and also to know quantitatively the avidity with which these salicylates bind metal ions.

Potentiometric titration appeared to be a suitable method for obtaining the desired information. Examination of the curves produced would indicate whether or not chelation had occurred, and stability constants could be calculated from the pH measurements. Mathematical treatment of the results was that derived by Albert (3, 4) for chelating agents having two ionizing groups. Babko (5, 6, 7) has previously obtained stability constants for salicylic acid with ferric, cupric, and aluminum ions using a spectrophotometric procedure based on the method of Job (8).

* Received August 21, 1959, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.

Abstracted from a thesis submitted by J. Pecci as a requirement for the degree of Master of Science, 1958.

This project was supported in part by a grant (A-1914) awarded by the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

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METHODS

Materials.—The metallic ions were used in the form of the following salts: Fisher certified $CuCl_2$, Baker analyzed reagents $Zn(NO_3)_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, $AlCl_3 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, $Ca(CH_3CO_2)_2 \cdot H_2O$, and $AgNO_3$. The *o*-hydroxybenzoic acid used was Fisher certified grade, the *p*-hydroxybenzoic acid obtained from Eastman Organic Chemicals, and the gentisic acid obtained from Matheson, Coleman and Bell. The salicyluric acid was prepared by the procedure of Hanzlik (9), m. p. 168–170°.

Carbonate-free 0.100 *N* sodium hydroxide was prepared from a filtered saturated solution of sodium hydroxide U. S. P. The stock solution was appropriately diluted with freshly boiled-out and cooled distilled water, and standardized.

Titrations.—Exactly 50.0 ml. of a 0.01 *M* solution of salicylic acid or derivative was titrated with 0.100 *N* sodium hydroxide, first in the absence of metal ions and then in the presence of 0.00025 mole of a divalent metal salt or 0.000165 mole of a trivalent metal salt (one equivalent). Thus the molar concentration was 0.005 *M* for divalent ions and 0.0033 *M* for trivalent ions, and the molar ratio of salicyclic acid to metal was 2:1 for divalent and 3:1 for trivalent ions. The total volume of the solution being titrated was therefore 50 ml., whether or not metal ions were present. The amount of alkali used in each titration was 10 ml.

Exactly 50.0 ml. of a 0.005 *M* solution of divalent

metal ions or 50.0 ml. of a 0.0033 *M* solution of trivalent metal ions was also titrated with 0.100 *N* sodium hydroxide. All titrations were carried out at 20 ± 2°.

The solution to be titrated was contained in a beaker fitted with a stopper admitting glass and calomel electrodes, a thermometer, buret tip, and capillary tube through which nitrogen was passed continuously for stirring and maintaining an inert atmosphere.

The alkali was added in 0.5-ml. portions and the pH was recorded after each addition by means of a Beckman model H2 pH meter frequently standardized with Beckman buffer solution. Graphs were then prepared by plotting pH *vs.* volume of alkali.

Expressions.—The calculations of stability constants are based on the following expressions, since chelate formation has been shown by Bjerrum (10) to be thermodynamically reversible without appreciable energy of activation and to follow a step-wise course, and the present results, as shown by the formation curves (Fig. 1), are in agreement.

$$K' = \frac{[1:1\ \text{chelate}]}{[\text{free metal ions}] \times [\text{free chelating species}]}$$

$$K'' = \frac{[2:1\ \text{chelate}]}{[1:1\ \text{chelate}] \times [\text{free chelating species}]}$$

By combining these two equations, an overall stability constant (Ks) for the entire reaction is obtained (where a divalent salt is involved):

$$Ks = \frac{[2:1\ \text{chelate}]}{[\text{free metal ions}] \times [\text{free chelating species}]^2}$$

or, more conveniently,

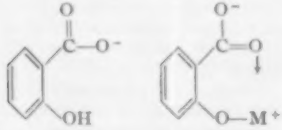
$$Ks = K'K''$$

In the case of a trivalent salt, the following expressions would, of course, be applicable:

$$Ks = \frac{[3:1\ \text{chelate}]}{[\text{free metal ions}] \times [\text{free chelating species}]^3}$$

$$Ks = K'K''K'''$$

The term "free chelating species" refers to that form of the chelating agent with which the chelate is in equilibrium. In the case of salicylic acid and a divalent metal ion the free chelating species in equilibrium with the 1:1 chelate is shown by structure I, and the free chelating species in equilibrium with the 2:1 chelate by structure II.



I II

Calculations.—If we define $\bar{n}$ as the average number of ligand molecules bound by each metal ion at any stage in the process of complex formation, and $[Sc]$ as the corresponding concentration of free chelating species, then K' and K'' can be calculated from Eqs. 1 and 2 without actual measurement of the free metal ion concentration (3, 10).

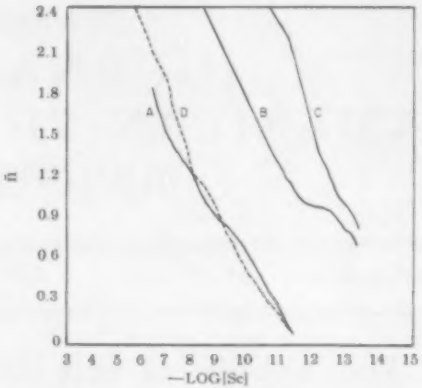


Fig. 1.—Formation curves of the salicylic acid chelates. *A*, Salicylic acid (0.01 *M*) and Cu^{++} (0.005 *M*); *B*, salicylic acid (0.01 *M*) and Al^{+++} (0.0033 *M*); *C*, salicylic acid (0.01 *M*) and Fe^{+++} (0.0033 *M*); *D*, theoretical curve for *A* assuming step-wise addition.

$$K' = \frac{\bar{n}}{(1 - \bar{n})[Sc]} \qquad \text{(Eq. 1)}$$

$$K'' = \frac{\bar{n} - 1}{(2 - \bar{n})[Sc]} \qquad \text{(Eq. 2)}$$

It follows then that K''' may be calculated from Eq. 3.

$$K''' = \frac{\bar{n} - 2}{(3 - \bar{n})[Sc]} \qquad \text{(Eq. 3)}$$

These equations can be solved because the experimental data allow the calculation of $[Sc]$ from Eqs. 4 or 5 and $\bar{n}$ from Eq. 6 as shown by Albert (4).

$$\log [Sc] = \log \{[XSc^\circ] - [\text{NaOH}] - [H^+]\} - \log \beta \qquad \text{(Eq. 4)}$$

$$\log [Sc] = \log \{2[XSc^\circ] - [\text{NaOH}] - [H^+]\} - \log \beta \qquad \text{(Eq. 5)}$$

$$\bar{n} = \frac{[XSc^\circ] - \alpha[Sc]}{[M^\circ]} \qquad \text{(Eq. 6)}$$

Where $[XSc^\circ]$ = the molar concentration of salicylate before metal ion was added (0.01 *M*); [NaOH] = the molar concentration of sodium hydroxide which would be present if salicylate and metal ion were absent; $[H^+]$ = the hydrogen ion concentration calculated from the pH readings; $[M^\circ]$ = the total molar concentration of metal ion, bound or free; $\alpha = ([H^+]/Ka) + ([H^+]/KaKa') + 1$; $\beta = ([H^+]/Ka) + (2[H^+]^2/KaKa') + 1$; Ka = the secondary ionization constant for the salicylate; Ka' = the primary ionization constant for the salicylate.

In order to decide whether to use Eqs. 4 or 5 to determine log $[Sc]$, a comparison was made of the pH values obtained on titration with alkali in the presence and in the absence of metal. For example, in Table I, salicylic acid in the presence of Cu^{++} ions shows evidence of end point formation when two equivalents (10.0 ml.) of alkali are added because the pH obtained in the presence of Cu^{++}

TABLE I.—POTENTIOMETRIC TITRATION OF SALICYLIC ACID AND CUPRIC CHLORIDE AT 20°

0.1 *N* NaOH, ml.	pH	log [*Sc*]	$\bar{n}$	log *K'*	log *K''*
0	2.62	...	...	...	...
0.5	2.68	$\overline{14}.77$	...	...	...
1.0	2.78	$\overline{14}.93$	...	...	...
1.5	2.88	$\overline{13}.09$	...	...	...
2.0	2.98	$\overline{13}.24$	...	...	...
2.5	3.12	$\overline{13}.44$	...	...	...
3.0	3.28	$\overline{13}.66$	0.03	...	...
3.5	3.42	$\overline{13}.84$	0.01	...	...
4.0	3.61	$\overline{12}.07$	0.02	...	...
4.5	3.88	$\overline{12}.38$	0.03	...	...
5.0	4.21	$\overline{12}.70$	0.10	10.34	...
5.5	4.58	$\overline{11}.07$	0.25	10.45	...
6.0	4.95	$\overline{11}.40$	0.45	10.52	...
6.5	5.32	$\overline{11}.73$	0.60	10.46	...
7.0	5.88	$\overline{10}.22$	0.80	10.38	...
7.5	6.88	$\overline{9}.14$	1.00	...	...
8.0	7.28	$\overline{9}.43$	1.18	...	7.91
8.5	7.62	$\overline{9}.66$	1.39	...	8.14
9.0	8.78	$\overline{8}.63$	1.60	...	7.54
9.5	10.31	$\overline{7}.85$	1.98	...	7.85
10.0	10.85	...	...	...	...
log mean of antilogs				10.43	7.91

ions is only 0.9 pH units below that of the pH of salicylic acid alone. At one equivalent (5.0 ml.), the pH of salicylic acid in the presence of Cu^{++} ions is 4.27 pH units below that of salicylic acid alone.

Whenever end point formation is reached after the addition of two equivalents of alkali, Eq. 5 is used. If end point formation had occurred after the addition of one equivalent of alkali, Eq. 4 would have been used. In this work, all values for log [*Sc*] were calculated from Eq. 5.

The overall stability constant was calculated from Eq. 7 and then compared with an independent calculation from Eq. 8.

$$\log Ks = -2 \log [Sc], \text{ where } \bar{n} = 1.00 \quad \text{(Eq. 7)}$$

$$\log Ks = \log K' + \log K'' \quad \text{(Eq. 8)}$$

When log *K''* could be calculated, log *Ks* was calculated from Eq. 9.

$$\log Ks = \log K' + \log K'' + \log K''' \quad \text{(Eq. 9)}$$

RESULTS AND DISCUSSION

Potentiometric titrations were carried out with salicylic acid in the presence of the following ions: Cu^{++}, Fe^{+++}, Al^{+++}, Co^{++}, Ni^{++}, Zn^{++}, Mg^{++}, Ca^{++}, and Ag^{+}. Examination of the curves drawn (pH *vs.* volume of alkali) showed that only copper, aluminum, and ferric ions were capable of chelating with salicylic acid. Gentisic and salicyluric acids also were found to chelate only these three metal ions of the metals studied. The titration curves obtained with the nonchelating metals in the presence of a salicylate reproduce in turn the titration curves of the individual components, whereas the titration curve representing a chelation, where additional hydrogen ions are liberated, follows "a path independent of that of the chelating agent and is almost always independent of that of the metal ion (3)." In the latter case, an entirely new picture is presented, and furthermore, the usual precipitates of metal hydroxides no longer take place upon the first addition of alkali. These differences in titration results from chelating and nonchelating reactions have been adequately illustrated in the past (3, 11), however, and need no elaboration here. The extent of the displacement of the curve representing a chelation forms a measure of the avidity of a particular agent for a given metal ion, and is dealt with algebraically in the section on calculations. It should be pointed out, however, that it is not easy to recognize a low value of *K'* by this method, so that a chelate of low stability could be overlooked.

In the titrations of the salicylates with various metal ions, pH values were obtained for each addition of alkali. From these readings, values for log [*Sc*] and $\bar{n}$ were calculated from Eqs. 5 or 6 and were used for the final calculation of stability constants. Table I gives a typical example of the results of a titration where chelation took place with a divalent metal ion and Table II where a chelation took place with a trivalent one. Values for log *K'*, log *K''*, and log *K'''*, calculated by Eqs. 1, 2, and 3, respectively, are included, as well as values for log *Ks* derived from both Eq. 7 and Eq. 8 in Table III. When log *K'''* could be calculated, Eqs. 7 and 8 could be used to calculate log *K'K''*, and log *Ks* was obtained from Eq. 9.

TABLE II.—POTENTIOMETRIC TITRATION OF SALICYLIC ACID AND FERRIC CHLORIDE AT 20°

0.1 *N* NaOH, ml.	pH	log [*Sc*]	$\bar{n}$	log *K'*	log *K''*	log *K'''*
0	2.25	...	...	...	...	...
0.5	2.30	$\overline{15}.98$	0.69	14.36	...	...
1.0	2.32	$\overline{15}.99$	0.82	14.67	...	...
1.5	2.40	$\overline{14}.14$	0.83	14.55	...	...
2.0	2.45	$\overline{14}.22$	0.88	14.65	...	...
2.5	2.52	$\overline{14}.33$	0.94	14.87	...	...
3.0	2.60	$\overline{14}.46$	0.99	...	...	...
3.5	2.69	$\overline{14}.60$	1.00	...	...	...
4.0	2.75	$\overline{14}.68$	1.10	...	12.39	...
4.5	2.85	$\overline{14}.82$	1.20	...	12.57	...
5.0	2.98	$\overline{13}.02$	1.21	...	12.41	...
5.5	3.10	$\overline{13}.17$	1.30	...	12.46	...
6.0	3.20	$\overline{13}.28$	1.40	...	12.54	...
6.5	3.30	$\overline{13}.38$	1.53	...	12.67	...
7.0	3.48	$\overline{13}.58$	1.60	...	...	...
7.5	3.60	$\overline{13}.66$	1.80	...	...	...
8.0	3.80	$\overline{13}.83$	1.99	...	...	...
8.5	4.08	$\overline{12}.04$	2.20	...	...	11.36
9.0	4.55	$\overline{12}.38$	2.44	...	...	11.52
9.5	5.80	$\overline{11}.36$	2.70	...	...	11.01
10.0	9.00	...	...	...	...	...
log mean of antilogs				14.65	12.52	11.35

The values obtained for log *K'*, log *K''*, and log *K'''* were converted to antilogarithms, averaged, and reconverted to logarithms. Not all values were selected for this purpose, as they are not all of equal accuracy. These results are summarized in Table III, and may be compared with literature *Ks* values for salicylic acid chelates in Table IV.

Curves obtained by plotting values of $\bar{n}$ against $-\log [Sc]$, as shown in Fig. 1, have been termed formation curves by Bjerrum (10), and they provide evidence as to whether a given reaction is stepwise or not. Figure 1 gives the formation curves for the chelates of salicylic acid with Cu^{++},

TABLE III.—STABILITY CONSTANTS OF THE SALICYLATES AND HEAVY METAL CATIONS AT 20°

Ionization Constants:	Salicylic Acid	Gentisic Acid	Salicyluric Acid
K_a	3.6×10^{-14} [a]	6.3×10^{-11} [a]	6.9×10^{-14} [d]
K_a'	1.05×10^{-3} [b]	7.9×10^{-4} [c]	2.3×10^{-4} [d]
Stability Constants:			
Cu^{++}			
log K'	10.4	7.0	6.1
log K''	7.9	4.9	5.6
log K_s [e]	18.3	11.9	11.7
log K_s [f]	17.7	11.6	11.4
Fe^{+++}			
log K'	14.7	10.8	9.0
log K''	12.5	8.0	7.0
log K'''	11.4	5.1	4.8
log K_s [g]	38.6	23.9	20.8
Al^{+++}			
log K'	14.0	10.4	7.9
log K''	10.7	7.3	5.7
log K'''	8.6	5.7	... [h]
log K_s [g]	33.3	23.4	18.0 [i]

[a] From Kolthoff, I. M., *Rec. trav. chim.*, **42**, 969(1923). [b] From "International Critical Tables," Vol. VII, McGraw-Hill Book Co., Inc., New York, N. Y., 1930, p. 246. [c] From Osol, A. and Kleckner, L. J., THIS JOURNAL, **41**, 306(1952). [d] From Kapp, E. M. and Coburn, A. F., *J. Biol. Chem.*, **145**, 549(1942). [e] From Eq. 8. [f] From Eq. 7. [g] From Eq. 9. [h] A precipitate occurred after the addition of 7.5 ml. of alkali. [i] Estimated value.

TABLE IV.—COMPARISON OF STABILITY CONSTANTS FOR SALICYLIC ACID CHELATES WITH LITERATURE VALUES

Cation	log K'	log K''	log K'''	log K_s [a]
Cu^{++} [b]	10.4	7.9	...	18.3 [c]
[d]	10.6	6.3	...	16.9
Fe^{+++} [b]	14.7	12.5	11.4	38.5
[e]	16.4 [f]	11.4	5.7	33.5
Al^{+++} [b]	14.0	10.7	8.6	33.3
[g]	14.0	9.0 [h]	3.0 [h]	26.0 [h]

[a] From Eq. 8 or 9. [b] Present work. [c] Using Eq. 7, log K_s = 17.7. [d] Ref. (5). [e] Ref. (6). [f] K' was found by Babko to be 4×10^{-12}. Log K' was reported to be 17.4, but obviously an oversight was committed. [g] Ref. (7). [h] Estimated value.

Fe^{+++}, and Al^{+++} ions. A theoretical curve was derived by adopting any arbitrary value for log K', log K'', and log K''' and working out values of $-\log [Sc]$ for regular increments of $\bar{n}$ from Eqs. 1, 2, and 3. The theoretical is seen to be parallel to the experimental curves, so evidence was obtained that step-wise addition had occurred and that the equations employed are valid. It should be pointed out that Eq. 7 is valid regardless of the tye of addition taking place because it is used only when $\bar{n} = 1.00$.

Another titration was carried out using *p*-hydroxybenzoic acid in the presence of Cu^{++} ions. Examination of the titration curve showed no evidence of chelation, and revealed a picture similar to that already obtained from the salicylates in the presence of nonchelating metal ions. The precipitate of metal hydroxide occurred at the same pH and the curve for the mixture of acid and metal reproduced the curves of the components.

From Table III, it is evident that neither gentisic nor salicyluric acid forms metal chelates as readily as salicylic acid. In fact, the overall stability constants (K_s) for salicylic acid-metal chelates are all greater than the constants determined by Albert (3, 4, 12) for the metal chelates of α-amino acids and other cellular constituents. It is quite conceivable, then, that in the cell, salicylic acid, or its metabolities, if present in sufficient concentration, may compete successfully with the naturally occurring substances for metal ions. It is also possible that the chelating ability of the salicylates may be of importance in their therapeutic effects, since metal chelation in the cell seems quite possible.

SUMMARY

1. Salicylic, gentisic, and salicyluric acids have been found to form stable chelates with Cu^{++}, Fe^{+++}, and Al^{+++} ions using potentiometric determinations. No evidence of chelation was observed with Co^{++}, Ni^{++}, Zn^{++}, Mg^{++}, Ca^{++}, or Ag^{+} ions.

2. The avidities with which these salicylates combined with heavy metal ions were calculated and recorded as stability constants. The constants obtained for salicylic acid with Cu^{++}, Fe^{+++}, and Al^{+++} ions agreed fairly well with those of Babko determined by a different method.

3. The following orders of chelate stability were found: salicylic > gentisic > salicyluric acid, and $Fe^{+++} > Al^{+++} > Cu^{++}$. In other words, the greater the secondary ionization constant of the acid, the smaller the value of the stability constant.

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Relationship of Chemical Structure to Central Nervous System Effects of Tranquilizing and Anticonvulsant Drugs*

By T. C. BARNES

Isolated mice provide a method of testing tranquilizing and anticonvulsant drugs on abnormal animals. Trifluoroperazine was effective in abolishing head twitch in lower doses than chlorpromazine, showing potency of the added piperazine ring. Phenobarbital inhibited convulsive movements induced by tactile stimulation but did not abolish head twitch.

PREVIOUS REPORTS in abstract form (1, 2, 3) have described the agitated behavior of mice and rats kept in solitary confinement. For effects in another species see (4). The method could provide a bioassay for tranquilizing and anticonvulsant drugs. Keller and Umbreit (5) have reported the violent twitch of the head produced by touching the occiput in an isolated mouse. Yen, *et al.* (6), observed that a mouse after isolation will attack a normal mouse. Pavlov (7) found that some isolated dogs become neurotic, refuse food, and die. Liddell (8) stated that only the sheep and goat can withstand isolation. Lasagna and McCann (9) investigated the increased toxicity of amphetamine in aggregated mice.

EXPERIMENTAL

In the experiments reported in this paper, white mice were taken from large communities and isolated in cages 6 × 6 × 12 inches, with metal sides and mesh top. Time of isolation lasted from twenty-four hours to a year. Head twitch was present in 5% of controls but only mice without this sign of agitation were used. Time of isolation required to develop twitch varied from several days to weeks (some mice did not acquire twitch).

Unlike Pavlov's (7) isolated dogs, isolated mice did not show anorexia and a full diet of fresh meat and vegetables was provided, besides the usual rat pellets. Lee, *et al.* (10), have shown that isolated mice become agitated sooner on vitamin B_{12}-deficient diet (the type of fat eaten is also a factor.) In our experiments anorexia occurred only with reserpine. Mice were isolated visually but could smell and hear the other mice. They were taken from stock cages containing a large number, to emphasize the factor of social isolation.

In mice, drugs were given orally with a blunt needle. Saline controls did not affect head twitch or convulsive movements. At least 40 mice were used for each drug (mice that had recovered from a drug were tested again with a different drug). The head twitch was produced by a light touch on the occipital region (tested every one-half hour over a seven-hour period). Doses were investigated from subliminal to those killing 5% or over (dead mice counted the next day). Mice were considered sedated if they showed slow locomotion when touched, or definite ataxia, or obvious sleep. Recording devices of the Skinner box type were not possible because the mouse had to be touched lightly on trigger zone for twitch (the occiput). Records of the spontaneous movements of normal mice as influenced by drugs are of less significance than the effects of drugs on abnormal movement of "neurotic" mice.

A convulsive movement, distinct from head twitch, is defined as a violent, purposeless displacement of the body (elicited by touching the occiput).

Twenty-five tame white rats were also subjected to visual isolation in cages with metal sides (same dimensions as for mice). These were mostly old rats (104–438 Gm). taken from a large community. The head twitch is not seen in the rat but agitation is shown by vicious attack on a blunt glass rod which originally did not elicit a pugnacious attitude. Some rats squealed, or rolled over, or showed catatonic postures after isolation from several days to months.

Agitated rats are too vicious to handle during administration of drugs by stomach tube so a method of self-administration of ether was used, which we developed previously for the study of wound healing (11). An inverted glass funnel containing cotton soaked in ether in the spout, was placed over the animal standing on a sheet of fly-screen resting on a flat board. When the rat tried to escape upward, it inhaled ether and fell to the ventilated floor, thus avoiding an overdose of anesthetic. The excitement stage of etherization was avoided. Rats also ate a rich diet (except on reserpine).

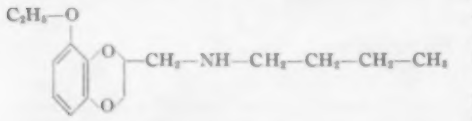
RESULTS

Table I presents the data obtained with 19 drugs on the head twitch and convulsive movements of isolated mice. The last column gives the percentage of mice which were sedated. The potency of head twitch inhibition decreased in the following order: chlorpromazine > promazine > Lilly 26125 > reserpine > trifluoperazine > acetazolamide > phenacemide > azacyclonol > ethotoin > meprobamate > glutamic acid lactam > primidone. The head twitch is perhaps a neurotic or psychotic sign. If so, the above compounds might be said to have tranquilizing action. On the other hand, trimethadione, methylsuximide, SKF 5627, SKF 2599, diphenylhydantoin, and phenobarbital did not inhibit the

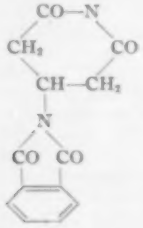
* Received October 12, 1959, from Hahnemann Medical College, Philadelphia, Pa., and Parmae Laboratories, Dallas, Tex.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

twitch. The minus sign before the phenobarbital data indicates that head twitch was increased. Drugs having no common name that would fit the first column are as follows: Lilly 56125 is 8-ethoxy-2-butylamino-methyl-1,4-benzodioxane.



SKF 5627 is 3-pthalmido glutaramide.



SKF 2599 is reduced diphenylhydantoin. It will be seen in Table I that the so-called convulsive movement or violent displacement of the body without purpose is inhibited by an anticonvulsive drug like trimethadione, which has no effect on the head shake. Dilantin increased the convulsive movements indicated by the minus sign in the table. The ethyl congener, ethotoin, and also the reduced forms of diphenylhydantoin did not increase convulsions. The twitch might be called a "psychomotor" phenomenon and it is of interest that phenacemide eliminated twitch in 19% of mice. However, methsuximide had no effect on twitch or convulsive movements but sedated 70% (40% of these died.) Note the dosage was carried above the 5% mortality level with this drug.

TABLE I.—EFFECT OF DRUGS ON AGITATED BEHAVIOR OF ISOLATED MICE[a]

Drug[b]	Oral Dose, mg./Kg.	Head Twitch Inhibited, %	Anti-convulsant Movement or Convulsion Stopped, %	Sedation, %
Chlorpromazine	3	70	90	20
Promazine	50	58	78	80
Lilly 26125[c]	12	50	87	60
Reserpine	10	45	47	54
Trifluoperazine	1	38	53	0
Acetazolamide	800	37	50	33
Phenacemide	1500	19	47	33
Azacyclonol	250	17	20	18
Ethotoin	400	15	33	77
Meprobamate	200	11	42	50
Glutamic acid lactam	800	11	58	24
Primidone	200	6	30	56
Trimethadione	800	0	53	27
Methsuximide	500	0	0	70
SKF 5627[c]	400	0	0	59
SKF 2599[c]	200	0	0	25
Diphenylhydantoin	200	0	−55	75
Phenobarbital	200	−14	60	85

[a] All experiments include 40 or more mice. Doses are optimum for inhibition of head twitch, data based on time for peak effect, 7-hour test intervals. If mouse reacted to tactile stimulus by violent displacement of body, movement was classed as convulsive.

[b] Drugs are listed in decreasing effect on head twitch; most effective action is that which inhibits the greatest percentage of mice with head twitch without producing more than 5% deaths.

[c] See text for chemical structure.

Another solvent which should be avoided is propylene glycol—at dose of 3,000 mg./Kg. inhibited twitch in 20% and sedation in 30% (no convulsive mice were available at the time.)

As a control for the phenothiazines, promethazine was tested at 25 mg./Kg. with no effect. To compare with the effects of acetazoleamide, chlorothiazide at 800 mg./Kg. was found to have no effect on behavior. As a control for Lilly 26125, phenoxybenzamine 1 mg./Kg. was without effect on twitch or convulsive movement. Statistical analysis by Dr. J. C. Munch on 16 drugs given in Table I (with the exception of promazine and trifluoperazine) shows that the coefficient of correlation r for antitwitch to anticonvulsant is 0.52, antitwitch to sedation 0.06, and anticonvulsant to sedation is 0.22.

Only 25 isolated white rats were studied so the results will be presented very briefly. Shortest time for developing aggressive behavior was twenty-nine hours, others remained tame for many months. Reserpine, oral suspension, was given at 1 to 5 mg./Kg. doses and prevented the attack on the glass rod in 50% of the agitated animals. In some, even after a total of 100 mg./Kg. reserpine over several days, the neurotic behavior persisted. Ptosis, hyena posture, and diarrhea resulted.

DISCUSSION

In experimental pharmacology, isolation in mice produces a head twitch which is inhibited by tranquilizing drugs. The effect of the added piperizino ring is seen in the lower dose of trifluoperazine compared with chlorpromazine. It is possible that a larger ring structure covers more receptors, protecting them from the stimulating action of neurohumoral agents. The 3 carbon and nitrogen sidechain at position 10 in the phenothiazines may attach to the same receptors that are used by the same structure in norepinephrine (12.) Once attached,

the inert rings would protect adjacent receptors. The antitwitch action of Lilly 26125 with benzodioxane structure suggests that an epinephrine-like compound may be involved in the agitated mice. However, this is not a simple matter of direct adrenolytic action since phenoxybenzamine is inactive. Possibly, drug must block adrenergic receptors in the nervous system, not in the vascular system. Depletion of norepinephrine in nerves by reserpine (13) is in accord with this hypothesis. However, the persistence of some aggression in rats after a total of 100 mg./Kg. of reserpine over several weeks, suggests that there are other factors besides chemical.

Minor technical points are the ptosis with reserpine which may aggravate the sensory deprivation or isolation stress and the chirping which occurs with 100 mg./Kg. promazine which may act in the opposite direction.

The diphenyl methane structure appears weak in agitated mice as judged by the effect of azacyclonol.

SUMMARY

1. Isolated mice provide a method of testing tranquilizing and anticonvulsant drugs.

2. Trifluoperazine was effective in abolishing head twitch in lower doses than chlorpromazine, showing potency of added piperazine ring.

3. Phenobarbital did not abolish head twitch in mice as did the tranquilizers. The reduced form had some antitwitch action.

4. The well-known exciting effect of diphenylhydantoin in mice is abolished by reduction or by ethyl congener.

5. Closing the structure of glutamic acid endows antitwitch and anticonvulsive properties.

6. Statistical analysis shows antitwitch effect distinct from anticonvulsive effect.

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Complexing Tendencies of Saccharin in Aqueous Solutions*

By J. R. MARVEL† and A. P. LEMBERGER

Contrary to what might be expected, relatively weak complexing tendencies were observed between saccharin and various substances in aqueous solutions. A 1 : 1 complex is formed between saccharin and theophylline. Saccharin forms water-soluble complexes with caffeine, various amides, and phenols. No interactions were observed between saccharin and N-methylpyrollidone, γ-butyrolactone and polyethylene glycol 4000.

IN RECENT STUDIES by Higuchi and co-workers (1–4) it has been shown that a variety of acidic compounds form molecular complexes with certain substances, such as caffeine, N,N,N′,N′-tetramethylphthalamide, polyvinylpyrrolidone, and polyethylene glycol 4000. Saccharin, which has been widely used as a synthetic sweetening agent for over a half century, possesses acidic properties and might be expected to show similar complexing behavior. However, Rogenbogen's report (5), in 1918, of an association between saccharin and antipyrine is the only literature citation of an organic complex formation involving saccharin.

Higuchi and Lach (4) have proposed that molecular interactions similar to caffeine complexes may be the result of an association between an acidic hydrogen and the electronegative center of the complexing agent. They also proposed that complexing may be enhanced by the high internal pressure of water which predisposes the interacting molecules to a stronger association. On the basis of this theory of complexing saccharin might be expected to show an attraction for compounds having a good electron donating center, such as the carbonyl group of an amide. It was the purpose of this study to investigate

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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

possible interactions between saccharin and various typical complexing agents.

The solubility method (1) was employed as the analytical procedure for the observation of the complexing tendency of saccharin with the investigated complexing agents. The solubility method was reported to be particularly applicable for detecting small complexing tendencies.

RESULTS AND DISCUSSION

Interactions With Amides.—All the investigated amides are freely soluble in water and except for N,N-dimethylacetamide, a high boiling liquid, are white crystalline solids. The observed solubility of saccharin in water at 30° with various concentrations of acetamide, N,N-dimethylacetamide, N,N-dimethylbenzamide, N,N,N′,N′-tetramethylfumaramide, and N,N,N′,N′-tetramethylphthalamide is shown in Fig. 1. The amount of saccharin used in these studies was 0.5 Gm. per 50 ml. of water.

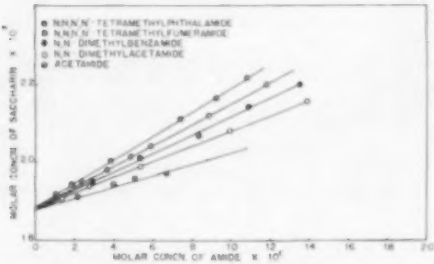

Fig. 1.—Solubility of saccharin in water at 30° as a function of amide concentration.

It was evident that saccharin associated with these amides to form soluble complexes. The linear relationship between the molar concentrations of the amide and the molar solubility of saccharin indicated a first-order dependency between the amide and the complex. Since all the amide complexes were found to be water soluble, it was impossible to calculate their actual stoichiometries. To facilitate a comparison of the complexing tendencies of saccharin with the amides, 1:1 stoichiometric relationships were assumed and overall stability constants for the complexes were calculated (6).

Since the ionization constant for saccharin is 2.5×10^{-2}, a considerable concentration of saccharate ions will be present in an aqueous solution of saccharin. It is possible that either undissociated saccharin or saccharate ion could associate with the complexing agents employed; therefore, the stability constants listed throughout this paper must be considered as overall stability constants.

The stability constants listed in Table I are considerably lower than the constants reported by Kostenbauder and Higuchi (2) in an investigation of N,N,N′,N′-tetramethylphthalamide and N,N,-N′,N′-tetramethylfumaramide with *p*-hydroxybenzoic acid and salicylic acid. It is evident that the extent of complex formation in the saccharin-amide systems is relatively small.

TABLE I.—OVERALL STABILITY CONSTANTS FOR INTERACTIONS OF SACCHARIN WITH CERTAIN AMIDES IN WATER

Amide	Stability Constant
N,N,N′,N′-Tetramethylphthalamide	1.6
N,N,N′,N′-Tetramethylfumaramide	1.4
N,N-Dimethylbenzamide	1.3
N,N-Dimethylacetamide	1.2
Acetamide	0.6

It is of interest to note that although only small differences in the stability constants are observed, the order in which they increase is compatible from a qualitative standpoint with theoretical expectations. The unsubstituted amide, acetamide, has been shown to form hydrogen bonds (7). If, as one might expect, such associations occur in aqueous solutions, they may well be in direct competition with the association between saccharin and amide. Alkyl substitution for amide hydrogen atoms precludes the possibility of formation of such hydrogen bonds. In addition, alkyl groups have a greater tendency to release electrons by the inductive effect than hydrogen atoms. With alkyl substitution the electronegativity of the carbonyl oxygen should be intensified and the ability of the amide to complex with an acidic substance should increase. As the complexing agents are rendered more hydrophobic, the "squeezing out" effect exerted by water molecules on a complex should be enhanced (4).

Interactions With Xanthines.—The effects of caffeine, theophylline, and theobromine on the solubility behavior of saccharin in water at 30° are shown in Fig. 2. The quantity of saccharin employed in these studies was 0.5 Gm. per 50 ml. of water.

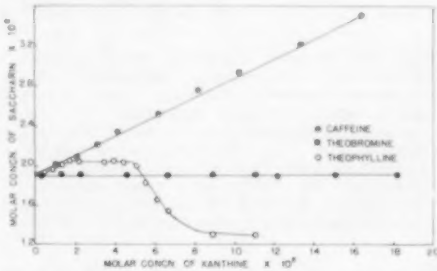

Fig. 2.—Solubility of saccharin in water at 30° as a function of xanthine concentration.

From the phase diagrams it is seen that caffeine and theophylline entered into definite complex formations with saccharin while theobromine apparently did not complex. Stability constants calculated from the initial linear portions of the phase diagrams gave values of 5.7 and 5.6 for theophylline and caffeine, respectively, when 1:1 interactions were assumed. Since the stability constants for the two xanthines are identical within experimental error, it appears that the caffeine and

theophylline interact with saccharin in a similar manner and differ only in the solubility of the complex.

The failure of theobromine to complex with saccharin may possibly be related to its structure. Since it is the 3,7-dimethyl derivative of xanthine, tautomerization involving the 1 and 6 positions can occur. Thus, if the 2 and 6 carbonyl functions are necessary for molecular interaction with saccharin, theobromine would not be expected to complex.

The solubility of saccharin in the presence of various concentrations of theophylline was increased until the solubility of the complex was reached. With a knowledge of the molar concentration of the xanthine corresponding to the plateau length in Fig. 2, the total amount of saccharin added to the system, and the total molar solubility of saccharin in the free and complexed forms, the calculated stoichiometric ratio was found to be 1:1.

Interactions With Other Substances Containing Nucleophilic Centers.—Within experimental error the solubility of saccharin at 30° in water was not increased in the presence of N-methylpyrrolidone, γ-butyrolactone, and polyethylene glycol 4000. The concentration range employed for these agents was 0.02 to 0.2 *M*.

Interactions With Phenols.—The study of the complexing tendency of saccharin with some slightly acidic substances was undertaken in view of the limited reactivity of saccharin with complexing agents which were proton-accepting agents.

The solubility of saccharin in water at 30° with various concentrations of phenol, hydroquinone, catechol, and resorcinol is shown in Fig. 3. It would appear that saccharin forms very weak complexes with these phenols. On the other hand, increases in solubility in the order of magnitude observed may be due, at least in part, to other phenomena. Assuming complex formation of a 1:1 nature for these proton-donating compounds with saccharin the stability constants fell into a range between 0.8 for phenol to 1.2 for resorcinol.

EXPERIMENTAL

N,N,N′,N′-tetramethylphthalamide, N,N,N′,N′-tetramethylfumaramide, and N,N-dimethylbenzamide were prepared by treating their acid chlorides in benzene with dimethylamine (2). The reaction system was extracted with water and the aqueous extract neutralized with sodium carbonate. The alkaline aqueous layer was extracted with chloroform and on evaporation of the chloroform, the amide was recovered. The amides were recrystallized from a mixture of equal volumes of benzene and ligroin.

Reagents.—N,N,N′,N′-tetramethylphthalamide, m. p. 122–123°; N,N,N′,N′-tetramethylfumaramide, m. p. 130–131°; N,N-dimethylbenzamide, m. p. 42–43°; acetamide, m. p. 81°; catechol, m. p. 104–105°; resorcinol, m. p. 110–111°; hydroquinone, m. p. 170–171°; phenol, reagent grade; caffeine, m. p. 236–238°; theophylline, m. p. 272–273°; theobromine, m. p. 357°; N,N-dimethylacetamide, Eastman Kodak (4972); N-methylpyrrolidone, General Aniline and Film Corp. (batch 503); γ-butyrolactone, Eastman Kodak (6922); 0.05 *N* sodium hydroxide solution; phenolphthalein and methyl red as indicators.

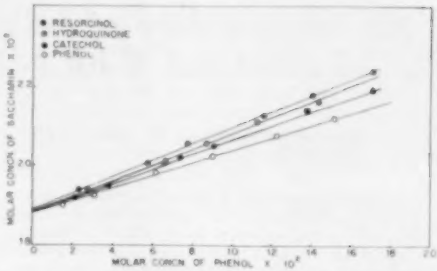

Fig. 3.—Solubility of saccharin in water at 30° as a function of phenol concentration.

Amides.—In 125-ml. glass-stoppered bottles excess quantities of saccharin were added to various quantities of amide in 50 ml. of water. The systems were equilibrated during rotation in a constant temperature bath at 30° for twenty-four hours. Aliquot portions of the supernatant liquid were removed and analyzed for saccharin content by titration with sodium hydroxide solution with phenolphthalein as indicator.

Xanthines and Phenols.—The procedure was identical to that described for the amides except methyl red was employed as the titration indicator with the phenols.

Polyethylene Glycol 4000, N-Methylpyrrolidone, and γ-Butyrolactone.—The general procedure with amide was varied in that the solubility of saccharin with these agents was determined by spectrophotometric measurements.

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An Investigation of Solvent Effect Upon Saccharin Complexes With N,N-Dimethylacetamide*

By J. R. MARVEL† and A. P. LEMBERGER

Complex formation between saccharin and N,N-dimethylacetamide appears to have a first-order dependency upon amide concentration in water, chloroform, and benzene. Relatively strong complexing occurs in benzene. In carbon tetrachloride and carbon tetrachloride-ethyl alcohol mixtures it appears that higher order complexes are formed. Increasing concentrations of ethyl alcohol decrease complexing tendencies through competition with N,N-dimethylacetamide for saccharin in solution.

IN A PREVIOUS communication (1), it was shown that saccharin formed weak complexes in aqueous media with certain amides, such as N,N-dimethylacetamide and N,N,N′N′-tetramethylphthalamide. Complexing of this nature was proposed by Higuchi and Lach (2) to be the result of dipolar interactions supplemented by the "squeezing out" effect by water molecules. Since saccharin possesses acidic hydrogen and these amides have good electron-donating centers, the results were somewhat unexpected.

Water is a strong dipole; thus, in an aqueous solution saccharin, its dissociation products, and an amide would probably be highly associated with water molecules. It was felt that in certain nonaqueous solvents where competing solute-solvent interactions would not be as significant the dipole-dipole interaction of saccharin and an amide might be rendered more favorable.

The present investigation is an observation of the complexing tendency of saccharin with N,N-dimethylacetamide in water, chloroform, benzene, carbon tetrachloride, and carbon tetrachloride-ethyl alcohol mixtures. The solubility method (3) was employed as the procedure for the detection of complex formation.

N,N-Dimethylacetamide was selected as the amide complexing agent because it exists only as a monomeric unit and possesses suitable solubility in the investigated solvents. This amide forms very weak complexes with saccharin in water; therefore, any increase in complexing tendency observed between this amide and saccharin in the nonaqueous solvents relative to water would be readily apparent.

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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

RESULTS AND DISCUSSION

Interactions in Benzene, Chloroform, and Water.—The effect of various concentrations of N,N-dimethylacetamide on the solubility of saccharin at 30° in benzene, chloroform, and water is shown in Fig. 1. The solubilities at 30° of saccharin in benzene, chloroform, and water are 4.3×10^{-3}, 2.04×10^{-2}, and 1.90×10^{-2} moles per liter, respectively.

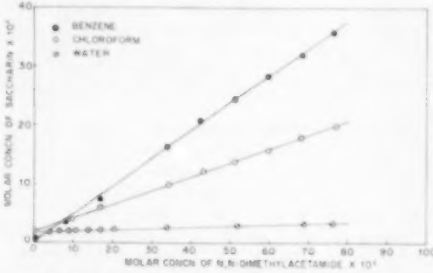

Fig. 1.—Solubility of saccharin in benzene, chloroform, and water at 30° in the presence of N,N-dimethylacetamide.

Since only soluble complexes are obtained in these solvents, the stoichiometry of the observed complexes can not be determined. However, the linear relationship which exists between the molar concentration of saccharin and the molar concentration of N,N-dimethylacetamide in each system appears to indicate that the principal complex has a first-order dependency with respect to the amide concentration. These linear relationships, although common to the three systems, do not necessarily indicate that the same complex is formed in all three solvents.

It is apparent that a quantitative comparison of the effects of these solvents upon the saccharin-amide complex would be difficult. Qualitatively the systems may be compared by determining the solubility of saccharin above its initial solubility for each system at a given amide concentration. Taking the increase in water solubility of saccharin by the amide as unity, a ratio of 31:16:1 is found for benzene, chloroform, and water, respectively. It is interesting to note that this ratio closely parallels the decrease in the polarity of the solvents. The ratios of the total molar concentration of saccharin to its initial molar solubility at 0.6 *M* N,N-dimethylacetamide are 66, 8, 1.5 for benzene, chloroform, and water, respectively. That is, the total saccharin in solution in the presence of 0.6 *M* amide in benzene is 66 times the solubility of saccharin in pure benzene. It would appear, then, that competitive interactions in aqueous systems inhibit saccharin-amide complex formation. Further, a

comparison of the results in benzene and chloroform indicates that in chloroform, solute-solvent interactions occur to reduce complex formation.

Interaction in Carbon Tetrachloride and Ethyl Alcohol-Carbon Tetrachloride Mixtures.—The solubility of saccharin in these solvents at 30° in the presence of varying concentrations of N,N-dimethylacetamide is illustrated in Fig. 2. The initial solubilities of saccharin are 0.82×10^{-3}, 1.68×10^{-3}, 3.16×10^{-3}, and 6.47×10^{-3} moles per liter in carbon tetrachloride, 5, 10, and 20% absolute ethyl alcohol by volume, in carbon tetrachloride, respectively.

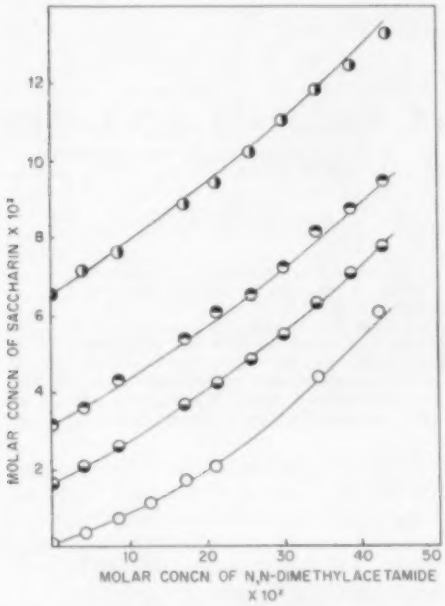

Fig. 2.—Solubility of saccharin in carbon tetrachloride-absolute ethyl alcohol v/v mixtures at 30° in the presence of N,N-dimethylacetamide. Curves are theoretical and points are experimental values. ○, 0% ethanol; ◒, 5% ethanol; ◓, 10% ethanol; and ◑, 20% ethanol.

The relationships in Fig. 2 between the molar solubility of saccharin and the molar concentration of amide indicate that significant amounts of more than one complex form in each system. Further, the relative amount of each varies depending upon which amide concentration is selected.

The interaction between saccharin and the amide complexing agent to form a complex may be expressed as

$$a \text{ saccharin} + b \text{ amide} \rightleftarrows c \text{ complex} \qquad \text{(Eq. 1)}$$

The stability constant for this reaction is then

$$K = \frac{(\text{complex})^c}{(\text{saccharin})^a\,(\text{amide})^b} \qquad \text{(Eq. 2)}$$

The total concentration of saccharin in solution, S_t, at any molarity of amide will be equal to the sum of the concentration of free saccharin, which is the solubility of saccharin, S_o, in the pure solvent, and the concentration of complexed saccharin. If it is assumed that two principal complexes are present in each system of Fig. 2, with the initial complex having a 1:1 and the second complex a 1:2 molecular ratio with respect to the saccharin and the amide, the total concentration of saccharin, as a function of free amide concentration, C, can be expressed mathematically as

$$S_t = S_o + K_1S_oC + K_2S_oC^2 \qquad \text{(Eq. 3)}$$

or

$$S_t - S_o = K'C + K''C^2 \qquad \text{(Eq. 4)}$$

where $K' = K_1S_o$ and $K'' = K_2S_o$.

Since complexing is weak in these systems, it may be assumed that the total concentration of the amide, C_t, is approximately equal to the concentration of the free amide.

Equation 4 can be rewritten in the following form

$$S_t - S_o = K'C_t + K''C_t^2 \qquad \text{(Eq. 5)}$$

The values for the constants K' and K'' were obtained by making a plot of $S_t - S_o/C_t$ against C_t. This plot gave a straight line with an intercept of K' and a slope of K''. The values obtained are tabulated in Table I and they were used to calculate the theoretical lines of Fig. 2. The points in Fig. 2 represent experimental values. It is recognized that in these solvents saccharin may be present as a dimer; however, the order in which the apparent values for K_1 and K_2 increase and their relative magnitudes should not be affected since the thermodynamic activity of saccharin is a constant.

TABLE I.—APPARENT STABILITY CONSTANT FOR ASSUMED 1:1 AND 1:2 COMPLEXES IN CARBON TETRACHLORIDE-ALCOHOL SOLVENT SYSTEMS

Ethyl Alcohol Content, % by Volume	K_1	K'	K_2	K''
20	2.0	0.13	1.2	0.08
10	3.5	0.11	2.5	0.08
5	6.0	0.10	6.0	0.10
0	73	0.06	220	0.18

The intent of the above calculations was not to obtain absolute values for K_1 and K_2 but rather to ascertain relative values by which the systems could be qualitatively compared. From these values, it would appear that the apparent complexing tendency between saccharin and N,N-dimethylacetamide increases as the competition from a component in the solvent decreases. As one might suspect in a competitive system, the effect of alcohol on the saccharin-amide complex appeared to be additive in the concentration range investigated. It would also appear that the alcohol content had a greater influence on K_2 than K_1.

EXPERIMENTAL

Reagents.—N,N-Dimethylacetamide, Eastman Kodak "white label" (4972); carbon tetrachloride, reagent grade; benzene, thiophene free; ethanol,

absolute, analytical grade; and sacharin U. S. P., recrystallized from acetone, m. p. 226–228°.

Procedure.—Excess quantities of saccharin were placed in 10-cc. Kimble clear-glass ampuls. Varying volumes of a standard amide solution and sufficient solvent to make 10 cc. were added. With benzene, prior to sealing, the ampuls were placed in a mixture of dry ice and acetone to solidify the contents. This procedure prevented loss of the solvent and reduced the fire hazard during the sealing operation. The sealed ampuls were agitated for forty-eight hours in a constant temperature bath at 30°. After equilibration, aliquot portions of the supernatant liquids were removed and diluted with 95% alcohol for spectrophotometric analysis at 285 mμ.

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The Preparation and Use of Radioiodinated Congo Red in Detecting Amyloidosis*

By MANUEL TUBIS, WILLIAM H. BLAHD, and ROBERT A. NORDYKE

Congo red concentrates in amyloid tissue. This study was undertaken to prepare a radioactive congo red which would concentrate in amyloid, permitting external counting and, thereby, scanning and localization, as well as blood concentration measurement. Two methods were used to trace-label the dye to an adequate specific activity for such studies. Injection into guinea pigs with amyloidosis resulting from induced chronic scurvy indicated deposits and radioactivity in the liver and other organs. The "tagged" dye shows promise for locating amyloid and may serve as a means for evaluating drugs for the resorption of amyloid. The method of direct radioiodination is applicable to other similar chemical compounds.

THE OBJECT of this study was to trace-iodinate Congo red so that the accumulation of the dye in amyloid tissue would permit external counting and blood disappearance measurements.

External counting would also permit the delineation of the deposits and provide a means for evaluating drugs which might cause a massive resorption of the deposits.

Amyloid is a carbohydrate, lipid, and protein complex which is deposited in vital organs following prolonged, infective, and tissue-destructive processes and may affect the structure and functions of these organs. Most typical amyloid is stained by Congo red and its fixation and retention is the basis for a presently used clinical test for amyloidosis. The test measures the unabsorbed circulating dye and requires several venipunctures and colorimetric determination of plasma concentration (1). These procedures would be eliminated by a satisfactory method of measuring blood concentration by external counting over a representative vascular bed, such as the side of the head.

* Received August 21, 1959, from the Radioisotope Service, Veterans Administration Center, Los Angeles, and the Departments of Radiology and Medicine, University of California Medical Center, Los Angeles, Calif.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

Two methods of labeling the dye were devised. The first was based on the method of Martin and Bang (2), who prepared tetraiodinated Congo red as a radiographic opaque medium using stable iodine. The second method was that of direct iodination of the dye using I^{131} and I^{127}.

A suitable test animal for *in vivo* localization was required and it had been reported (3, 4) that guinea pigs maintained on subminimal daily amounts of vitamin C low enough to produce chronic scurvy, develop amyloidosis. The distribution of congo red in such animals as well as in normal guinea pigs was studied.

EXPERIMENTAL

Synthesis I of I^{131}-labeled Congo red was based on that of Martin and Bang (2). Benzidine was trace-iodinated using sodium radioiodide, potassium iodide, and iodine in acid solution. The iodinated benzidines were then diazotized and coupled with sodium naphthionate to give a trace-iodinated Congo red.

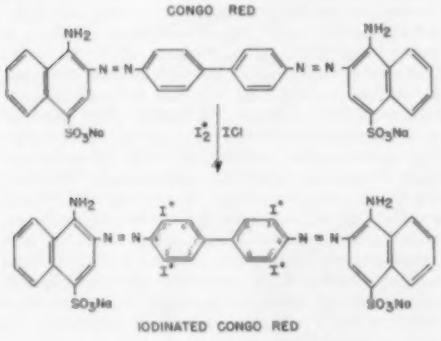
In a typical preparation, 384 mg. benzidine hydrochloride yielded 291 mg. of mixed, trace-iodinated benzidines with a specific activity of 2.59 μc./mg., representing a 4.5% utilization of the original 20 mc. I^{131} used, corrected for two days decay during preparation. The product was a purplish colored crystalline mass which did not

melt at 300°; insoluble in water, carbon tetrachloride, or acetic acid but soluble in methanol and ethanol.

The labeled Congo red was prepared by diazotizing the radioiodinated benzidines and coupling with sodium napththionate. The dye was extracted and when finally purified had a specific activity of 0.22 μc./mg. The iodine content by the Kingsley and Schaffert micromethod (5) was 17.5% whereas the iodine content of a tetraiodinated Congo red would have been 42.3%, as reported by Martin and Bang (2). This indicated that our Congo red had, on the average, only 1 atom I per molecule. The yield of finally purified dye was only 34 mg. or 8.9% of the iodinated benzidines used and the activity represented only 0.4% of the original I^{131} activity used, corrected for decay.

Synthesis II of I^{131}-Labeled Congo Red.—In order to obviate the necessary shielding and manipulation as well as the time-consuming procedures which resulted in low yields and loss of activity of radioiodine, a second synthesis was devised. Inasmuch as the same positions in the benzidine moiety of the dye were assumed to be available for iodination, "direct iodination," using I^{131}, of the pure dye was tried, and found to yield a pure product of high specific activity, capable of rapid preparation and purification. In this method, congo red U. S. P. in alcoholic suspension was iodinated with a chloroform solution of I^{131} of high specific activity in the presence of iodine monochloride and the final dye washed with appropriate solvents, after which adsorbed iodine was removed by dialysis or anion exchange. This reaction is shown in the following equation:

SYNTHESIS II



The radioiodine is shown to be in the 3, 3′, 5, and 5′ positions but since it is trace-iodinated, it may be in any one or more of these positions in any of the molecules.

A typical preparation was as follows: 85 mg. of Congo red U. S. P. was suspended in 30 cc. absolute ethanol, in a 100-cc. round-bottom flask connected by a ground-glass joint with a reflux condenser. In a 25-cc. Squibb-type separator were placed 2 cc. of water-washed chloroform which had been dried over anhydrous sodium sulfate. To the funnel were then added 0.33 cc. of 0.01 *M* potassium iodide solution, 0.20 cc. of 2.5 *M* hydrochloric acid, and 0.10 cc. of *M* sodium nitrite and approximately 40 mc. I^{131} solution in a volume of 1 to 3 cc. The funnel was swirled to mix the aqueous layer, then shaken vigorously to extract the liberated I^{131} and I^{127} by the chloroform. The chloroform solution was drained carefully into another similar funnel, avoiding any of the aqueous supernate, and the addition of similar quantities of the reagents except the I^{131} was repeated. The funnel again was shaken and the second chloroform extract combined with the first, and washed with two or more 2-cc. portions of water until the pH was not less than 5 to 5.5 (test paper). The chloroform solution was added to the flask containing the Congo red and to this was added 0.2 cc. of a chloroform solution containing 0.0163 mg. of iodine monochloride. The condenser was attached and the contents actively refluxed in a water bath for eight to twelve hours. At the end of this time, the condenser was removed and the solvents evaporated nearly to dryness in a well ventilated hood. The pasty residue was loosened and transferred to a small M-porosity sintered-glass filter with the aid of 20 cc. of water-washed, dried chloroform and further washed with 20 cc. of a mixture of three volumes of ethyl ether and one volume absolute ethanol, saving all washes for decay and safe disposal. The dye was air dried. The recovery was practically quantitative.

The dye contained small quantities of adsorbed I^{131} which were removed by dialysis *vs.* multiple 4-L. changes of distilled water adjusted to pH 7.2 to 7.6 and containing about 1 mg. sodium iodide. Dialysis was continued until a 5-cc. portion of the dialysate showed a count of one to two times that of background, usually requiring 10 or so dialysis periods of one hour each. The per cent dialyzable I^{131} of the final dye was *ca.* 0.5% of the total activity.

An alternate, preferable procedure for the removal of adsorbed I^{131} was the use of a column of anion exchange resin Amberlite IRA-400[1] in either the OH or Cl form, analytical grade, 30–50 mesh. The resin was prepared by first washing until the wash water was color free, then completely saturating with dye by soaking in 1% Congo red U. S. P. for several days. The column was made of 4-mm. glass tubing i. d., 35 cm. long, and contained *ca.* 1 Gm. of the dried, prepared resin. The column was washed with 1% Congo red solution just prior to use and then the labeled dye in 1% concentration passed through. No abstraction of labeled dye occurred. A single passage removed unbound I^{131} iodide as evidenced by later dialysis, ascending paper chromatography in 70% alcohol, and radioautography. The pH of the effluent labeled dye was 9.5 after use of the IRA-400 (OH) form and 7.1 to 7.5 after IRA-400 (Cl) form and the pH was adjusted to meet U. S. P. XV requirements of Congo red injection, i. e., 7.0 to 9.0. The dye solution was sealed in serum vials and sterilized in boiling water for thirty minutes.

The acute intravenous toxicity LD_{50} was 3.8 mg./20 Gm. mouse, as determined by an independent laboratory in a manner similar to that of Somers and Whittet (6).

By the direct iodination method, in one run, the

[1] Product of Rohm and Haas Co., Philadelphia 5, Pa. Available from Fisher Scientific Co., Pittsburgh, Pa.

specific activity of the final purified dye was 22.1 μc./mg. and the iodine content was 0.52% (5), indicating a ratio of 1 atom iodine per 35 molecules of dye. In this run, the final weight of unpurified dye was 91.4 mg. obtained from 85 mg. Congo red originally used. This represented a utilization of 5.2% of the I^{131} used, when corrected for decay. The average specific activity of the resin-purified material was 9.7 μc./mg. The purified dye was found to have the identical absorption maximum, i. e., at 500 mμ, solubilities and chromatographic behavior as the unlabeled dye.

Studies of Distribution in Scorbutic and Normal Guinea Pigs.[2]—Male guinea pigs weighing from 200 to 400 Gm. were divided into groups of normals and those to be fed a chronic scorbutogenic diet. The normals were fed *ad libitum* an adequate diet consisting of Rockland guinea pig diet ("C" Fortified)[3] which contained "vitamin C fortified to guarantee 8 mg. daily" per pound. In addition, these controls were given adequate daily supplements of vitamin C orally, approximately 6 mg. The daily weight gain was 4 to 6 Gm. and they thrived.

The "scurvy" guinea pigs were fed *ad libitum* a scorbutogenic diet of Rockland rabbit ration[3] which contained "vitamin C, inherent trace." In addition, they were given vitamin C orally, 0.1 to 0.2 mg. in one series and 0.4 to 1.0 mg. in a later series, to maintain the animals in the chronic stage of the disease.

When the vitamin C-deficient guinea pigs showed one or more of the symptoms of scurvy, they were maintained for periods of one to three weeks so as to prolong the disease and allow the development of amyloidosis. The average number of days on the scorbutogenic diet was twenty-four in one series and thirty-eight in a later series.

When the animals were considered suitable for the test, the normal and chronically scurvy animals were weighed and injected intraperitoneally with Nembutal[4] at the dosage of 12 mg./454 Gm. followed by the labeled Congo red at the dosage of 1 mg./454 Gm. body weight, the human dosage suggested by Unger, *et al.* (1). The doses administered varied from 0.43 to 1.29 mg., in volumes of 0.11 to 0.42 cc., and represented 0.96 to 8.82 μc. These were injected into a femoral or visceral vein after exposure. Later, it was found more convenient to inject via a lateral vein in the penis. This prevented contamination of the viscera and counting of this organ showed that very little of the dose remained at the site of injection. Serial blood samples were taken from the heart or hepatic veins at early times of the order of two to five minutes after injection, and thereafter at intervals such as ten, twenty, and thirty minutes, and at sacrifice, which was thirty to forty minutes for the first series and forty to fifty-five minutes for the second series. The guinea pigs were killed by exsanguination, and at post-mortem the organs were removed and weighed. Their gross appearance was noted and sections were taken for microscopic studies as well as for weighing in capped counting tubes for radioactivity estimations in a well-type scintillation counter.[5] The radioactivities of the organs and fluids were calculated on the basis of counts/minute/Gm. or counts/minute/cc. From these values were calculated the per cent of injected dose for normal and scorbutic guinea pigs in Table I.

A summary of the qualitative difference between normal and scorbutic animals would be as follows: the distribution of labeled Congo red is generally in the same range with considerable overlap except for liver, lung, and thyroid which seems to be about twice as high in the scorbutic.

In some of the scorbutic animals, the livers were perfused and retained their radioactivity despite the removal of the blood. Grossly, sections were visibly stained by the dye which was more visible in long-time frozen slices. The spleen and adrenals of the scorbutic animals were usually enlarged and abnormal in texture but had only as much activity per Gm. as the normals. The liver of the scorbutic animal had a greater activity than the normal, but the kidney activity was of the same order. According to Anderson (7) the adrenals and spleen, as well as liver and kidney, are the organs most frequently the site of deposition of amyloid. Richardson (8) using very much larger doses of unlabeled Congo red in rabbits and cats indicated that the dye in "the liver, spleen, lungs, kidneys, skin, and so on, is partially bound, physically or chemically, by some cellular or noncellular constituent, otherwise its concentration would remain in equilibrium with that of the blood." This is confirmed by our data.

A comparison of the clearance of our labeled dye from the blood of normal and scorbutic animals shows some variance with that reported in the literature. Both our normal and scorbutic animals showed about 80% removal from the blood between thirty-five to fifty-five minutes. This is in general agreement with Knorpp, *et al.* (9), who prepared Congo red labeled with S^{35} and used this in human subjects and reported 85% removal in ten to thirty minutes, indicating that injected small amounts are removed rapidly. Richardson (8) showed that rabbits injected intravenously with 50 mg./Kg. cleared approximately 25% from the blood in the first hour, despite this high dosage level. This agrees with Bennhold (10) who reported a 20% removal in man in the first hour, with a dosage level of 100 mg. Unger, *et al.* (1), using a dosage level of 1 mg. per pound of body weight in normal human subjects, reported 14% removal in the first thirty minutes, and 76% removal in the case of amyloid patients.

The variance of our results from those of Richardson (8) may be explained, aside from a species difference, by the dosage levels, wherein he administered 23 mg. per pound of body weght to rabbits and 204 mg. per pound to cats. Our dosage level of 1 mg. per pound, the common human dosage, might be considered "physiological" and did not cause "overloading" of the excretory systems.

[2] The authors wish to acknowledge gratefully the expert technical assistance of Mr. Edward Posnick and Mr. Laurence V. Johnson in these studies.

[3] Distributed by A. E. Staley Mfg. Co., Decatur, Ill.

[4] Abbott Laboratories, North Chicago, Ill.

[5] Tubes used were screw-cap culture tubes 16 × 125 mm. The crystal used was 2 × 2 inches, sodium iodide, thallium activated. The scaler was SC-51 Autoscaler, Tracerlab, Boston, Mass., and had four SC-41T binary plug-in units. The input circuit had a 1 microsecond resolving time. In a 5-cc. volume, samples had *ca.* 700,000 counts/minute/microcurie of I^{131}.

TABLE I.—PER CENT INJECTED DOSE OF CONGO RED-I^{131} PER GM. OF TISSUE OR CC. OF FLUID

Tissue	Normal		Scorbutic	
	Average	Range	Average	Range
Blood 2′–5′[a]	1.65(3)[b]	1.50–2.17	1.63(8)	0.92–2.74
Blood 30′–40′[a]	0.78(4)	0.57–1.16	0.78(8)	0.59–1.22
Liver	1.76(4)	0.35–2.95	4.31(10)	2.03 –7.28
Kidney	0.42(4)	0.22–0.61	0.37(10)	0.20 –0.61
Spleen	3.26(4)	1.27–6.22	2.08(10)	0.39 –5.36
Adrenals	0.53(4)	0.32–0.73	0.68(10)	0.26 –1.92
Thyroid	0.21(3)	0.10–0.28	0.44(8)	0.11 –1.50
Brain	0.03(2)	0.03	0.04(4)	0.03–0.05
Bile[a]	0.85(4)	0.39–1.41	1.40(7)	0.14–3.57
Lung	0.61(2)	0.54–0.68	1.03(5)	0.47–1.80
Leg muscle	0.08(2)	0.06–0.10	0.09(5)	0.007–0.20
Heart	0.30(2)	0.24–0.35	0.30(5)	0.16–0.38
Urine[a]	0.13(4)	0.01–0.32	0.06(8)	0.002–0.27

[a] Fluids, values given per cc.
[b] The number in the parentheses is the number of animals whose tissues were averaged.

The use of labeled Congo red provided a means of confirming the observations of others regarding the urinary elimination of the dye. Harmon, *et al.* (11), reported urinary excretion following intravenous injection. Unger (1), on the basis of an accurate extraction procedure, found only a faint trace of the dye. Richardson (8) reported a slight undetermined amount in rabbit urine and none in cat urine. Reference to Table I shows that variable, small but discrete amounts of activity are eliminated via the urine. That this activity is not due to liberated I^{131} from metabolized dye is borne out by the fact that normal guinea pigs injected with an equal number of counts of NaI^{131} showed a urinary activity greater than any of the dye-injected normal or scorbutic animals. Additional confirmation that the activity in urine was not due to liberated I^{131} is borne out by the relatively low count in the thyroid glands of dye-injected guinea pigs compared with those animals injected with the same number of counts of I^{131}, in which the urine was higher than the average of all the experimental animals on labeled dye, and very many times higher than most. The thyroid count of the animals injected with I^{131} was 4 to 20 times higher than either the normal or scorbutic animals injected with labeled dye. Incidentally, reference to Table I shows that the thyroid of the scorbutic animals was twice that of the normals. Our results confirm Richardson (8), that a blood-brain barrier to the dye seems operative.

Histological examination of the kidney and liver samples of some of the scorbutic animals indicated very slight deposits of amyloid-like substance which took the specific stains.

DISCUSSION

The use of trace-labeled Congo red permits physiological studies of the localization of the dye in amyloid tissue as well as its normal distribution and excretion. It was felt by the authors that one of the reasons that the tetraiodinated Congo red of Martin and Bang (2), who attempted to use this as a radiopaque substance, may have failed to localize sufficiently in amyloid tissue, was due to the changed physiological behavior of the heavily iodinated molecule. Our clearance rates from the blood are similar to those of Knorpp (9) whose congo red was only trace labeled with S^{35}.

The guinea pig was chosen for these experiments because it was felt that the induced chronic scurvy and resultant amyloidosis would make it an almost ideal test animal. However, it has been shown that only when the scurvy is prolonged is the formation and deposition of amyloid accomplished. Mice injected subcutaneously with solutions of sodium caseinate (12) over periods of several months develop amyloidosis and these are being prepared for confirmatory studies.

SUMMARY

Two syntheses for the preparation of labeled Congo red containing I^{131} have been described. The identity and purity has been established by spectrophotometry, chromatography, and radioautography. The method of "direct iodination" was applied successfully to trypan blue(13). Studies were made of the blood disappearance, tissue distribution, and excretion in normal and scorbutic guinea pigs. The feasibility of external counting as a means of measuring blood disappearance and localization has been demonstrated. Some of the observations regarding the distribution of the dye have been confirmed.

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An Investigation of *Coprinus atramentarius* for the Presence of Disulfiram*

By J. K. WIER† and V. E. TYLER, Jr.

Coprinus atramentarius Fries was grown in surface culture on selected media. The quantity of mycelium formed was small; no carpophores were produced. Addition of a low concentration of disulfiram to cultures of the fungus had no apparent effect on the development of the organism, and the added disulfiram disappeared from the cultures within sixty days. Concentrated extracts of the media and mycelia of the cultures of the fungus, as well as extracts of naturally-occurring carpophores, were investigated for the presence of disulfiram by means of a paper partition chromatographic method. It is concluded that the disulfiram-like physiological activity which was exhibited by the naturally-occurring carpophores of *C. atramentarius* is due to some agent other than disulfiram.

IT HAS LONG been known that ingestion of the mushroom *Coprinus atramentarius* Fries and the subsequent ingestion of alcohol gives rise to physiological symptoms very similar to those of the alcohol-disulfiram syndrome. Reports of *Coprinus*-alcohol poisoning are common in the mycological literature (1–5) and antedate considerably the discovery of the alcohol-disulfiram syndrome. The remarkable similarity between the two types of poisoning has given rise to considerable speculation that disulfiram might exist in *C. atramentarius* (4).

In 1956 two Czechoslovakian investigators, J. Simandl and J. Franc (6), reported the isolation of disulfiram from *C. atramentarius*. They extracted autolyzed carpophores of the fungus with carbon tetrachloride in the cold, evaporated the carbon tetrachloride extract, and extracted the resulting residue with anhydrous methanol. Evaporation and recrystallization yielded a crystalline substance with a melting point of 70.5°, the melting point of disulfiram, and this substance had the properties of disulfiram when subjected to paper partition chromatographic procedures. They did not attempt a quantitative determination.

Since the physiological action of the combination of alcohol and *C. atramentarius* suggested the possible presence of disulfiram in that fungus, and since the presence of disulfiram in the fungus had been reported by Simandl and Franc, it seemed of interest to determine if quantities of the organism could be produced successfully in culture and if the fungus would produce disulfiram when so grown. The culture of *C. atramentarius* used to initiate this investigation was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Although numerous hymenomycetes, including a number of *Coprinus* species, have been successfully grown in the laboratory, a recent review (7) revealed that the culture of *C. atramentarius* had been studied by only one investigator, C. H. Chow (8).

A number of media have been employed for culture of *Coprinus* species (9–13). Fresh horse dung or extract of fresh horse dung, alone or in combination with other nutrients, was the substrate most commonly used. Chow used fresh horse dung as well as other media in his cultures of *C. atramentarius*. He maintained only a few cultures of this fungus, as the spores germinated with difficulty and in low percentage, and he reported that the organism did not produce normal carpophores in artificial culture.

During the course of the present investigation it became obvious that *C. atramentarius* was not producing disulfiram when grown on the media employed, and it was decided to investigate the effect on the development of the fungus of adding disulfiram to the culture medium.

Locally-occurring carpophores of *C. atramentarius* were also investigated for presence of disulfiram. In the spring of 1957 a case of typical *Coprinus*-alcohol poisoning had occurred in Seattle, Washington (14). The victim, R. Levin, a University of Washington student, obtained the mushrooms which he ate from the exact site as that from which the mushrooms employed in this investigation were obtained. The growth habit of *C. atramentarius* is such that all these carpophores may be presumed to have arisen from the same mycelial source. Identification of the naturally-occurring carpophores of *C. atramentarius* employed in this investigation was made by Professor D. E. Stuntz, Department of Botany, University of Washington.

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† Abstracted from a dissertation submitted to the Graduate School of the University of Washington by J. K. Wier in partial fulfillment of the requirements for the degree of Master of Science.

EXPERIMENTAL

Culture of the Organism.—Seven media were originally employed for culture of *C. atramentarius* on a small scale. These were either media used previously for culture of macrofungi by other investigators or modifications of such media made by the authors in an attempt to improve the development of *C. atramentarius*. The media employed were the following:

Medium No. 1

Mannitol	2.0%
Casein hydrolysate	1.0%
$FeSO_4 \cdot 7H_2O$	0.01%
Basic nutrient solution (15)	10.0%
Distilled water	*q. s.*

This medium was used for the culture of various strains of *Claviceps purpurea* (15), and it has recently been employed for the culture of *Panaeolus campanulatus* by V. E. Tyler, Jr. (16).

Medium No. 2

Glucose	2.0%
Casein hydrolysate	1.0%
$FeSO_4 \cdot 7H_2O$	0.01%
Basic nutrient solution (15)	10.0%
Distilled water	*q. s.*

Medium No. 3

Glucose	2.0%
Casein hydrolysate	1.0%
Yeast extract	1.0%
$FeSO_4 \cdot 7H_2O$	0.01%
Basic nutrient solution (15)	10.0%
Distilled water	*q. s.*

Medium No. 4

This consisted of Medium No. 3 to which thiamine hydrochloride (200 mcg./L.) was added.

Medium No. 5 (Modified McCrea's medium)

Maltose	1.25%
Peptone	0.125%
Yeast extract	1.25%
KH_2PO_4	0.25%
$MgSO_4 \cdot 7H_2O$	0.125%
Distilled water	*q. s.*

This medium has been employed for the culture of *Claviceps purpurea* (17) and gave satisfactory development of *C. atramentarius* in slant tube cultures.

Medium No. 6 (Modess medium)

Glucose	0.5%
Malt extract	0.5%
KH_2PO_4	0.05%
$MgSO_4 \cdot 7H_2O$	0.05%
NH_4Cl	0.05%
$FeCl_3$	0.005%
Distilled water	*q. s.*

This medium has been used by the Centraalbureau voor Schimmelcultures for the culture of *Boletus luridus* and *Amanita muscaria* (18).

Medium No. 7

Maltose	0.5%
K_2HPO_4	0.025%
$MgSO_4 \cdot 7H_2O$	0.05%
$Ca(NO_3)_2 \cdot H_2O$	0.05%
Horse dung extract	33.33%
Distilled water	*q. s.*

The horse dung extract was prepared by boiling 1 Kg. of fresh horse dung with 1 L. of water for five minutes, straining, and filtering the product, and making to a volume of 1 L. with water. This medium was used by Bille-Hansen (12) for the culture of a number of *Coprinus* species.

For the small scale cultures, 125-ml. portions of the various media were introduced into Roux-type culture flasks. These flasks of media were then sterilized by autoclaving, cooled, and inoculated with the fungus. The inoculated flasks were placed in a constant temperature cabinet maintained at 25° ± 1°. The cabinet had glass doors, and the cultures were exposed to the indirect artificial illumination of the room in which the cabinet was located. The most rapid growth of the organism occurred in medium No. 7, the horse dung extract medium, and it was selected for routine use.

Subsequent cultures were maintained in both the Roux-type culture flasks and in diphtheria toxin culture bottles, the latter containing 1,200 ml. of medium. These cultures were also kept in the constant temperature cabinet. All cultures were harvested when visible growth of mycelium had ceased, a period of sixty to ninety days.

The medium of each culture was separated from the mycelium by straining through a coarse mesh sieve and was then processed to determine the presence or absence of disulfiram. The mycelium so collected was placed in porcelain evaporating dishes and dried in a forced air oven at 45° for three days. The mycelia from cultures grown on the same medium and inoculated on the same day were combined and weighed. The dried mycelium was then investigated for presence of disulfiram.

The development of the fungus was very sparse in all the media. Table I presents a summary of data on the growth of the fungus in the seven media, and the ratios of weight of dried mycelium to volume of medium.

TABLE I.—MYCELIAL YIELDS OF *Coprinus atramentarius* OBTAINED IN DIFFERENT MEDIA

Medium No.	No. of Cultures	Volume of Medium Processed, L.	Total Weight Dried Mycelium, Gm.	Dry Weight of Mycelium, mg./ml. of Medium
1	12[a]	1.500	0.99	0.66
2	8[a]	1.000	0.75	0.75
3	8[a]	1.000	0.82	0.82
4	8[a]	1.000	0.64	0.64
5	16[a]	2.000	1.47	0.74
6	8[a]	1.000	0.65	0.65
7	19[a]	2.325	2.40	1.03
7	13[b]	15.600	12.05	0.77

[a] Roux flask cultures. [b] Diphtheria toxin flask cultures.

To determine whether disulfiram had any effect upon the growth of *C. atramentarius*, twenty 250-ml. Erlenmeyer flasks containing 75 ml. of medium No. 7 to which 0.167 mg. % of disulfiram had been added were inoculated with the fungus. The concentration of disulfiram which could be added was limited by its extremely low water solubility. Another series of 10 flasks of medium No. 7 to which no disulfiram was added was inoculated with the fungus.

An additional 10 flasks of medium No. 7 containing the disulfiram were prepared but not inoculated. The two series of flasks which had been inoculated with the fungus were harvested at the end of sixty days. The total weight of dried mycelium from the 20 cultures with added disulfiram was 2.55 Gm. (1.70 mg. mycelium per ml. of medium). The total weight of the dried mycelium from the 10 cultures with no added disulfiram was 1.30 Gm. (1.73 mg. mycelium per ml. of medium). The added disulfiram apparently had no effect on the development of the fungus.

Five of the 10 flasks of uninoculated medium containing added disulfiram were tested for the presence of that compound shortly after they were prepared. The other five flasks of medium in that series were similarly tested after sixty days. Disulfiram was detected in both cases. Apparently prolonged solution in the medium did not cause significant decomposition of the disulfiram. Neither the medium nor mycelium of the 20 cultures to which disulfiram had been added contained detectable quantities of disulfiram at the end of the sixty-day growth period. The fungus had apparently decomposed the added disulfiram.

When medium No. 7 which had been solidified by the addition of 2% agar was inoculated with the fungus, a sparse feathery growth of mycelium occurred. Small darkened areas of mycelial compaction occurred which exuded a small amount of liquid similar in appearance to the honeydew of ergot. No carpophores were produced.

Detection of Disulfiram.—Disulfiram was separated from semipurified extracts by paper partition chromatography and rendered visible on the sheets by treatment with a suitable reagent. An attempt to utilize the general procedure of Divatia, Hine, and Burbridge (19) for the spectrophotometric determination of disulfiram in blood proved unsuccessful when applied to disulfiram in medium No. 7.

The chromatographic procedure chosen was a modification of that employed by Simandl and Franc (6). Sheets of Whatman No. 1 filter paper, 22 cm. × 56 cm., were immersed for a few seconds in a mixture of kerosene and benzene (1 to 9). The sheets were allowed to air-dry for five minutes and were then reimmersed in the kerosene-benzene mixture. After another five minute drying period, spots of the solutions to be investigated were applied along a line 12 cm. from one end of the kerosene-impregnated sheets of paper. Total amounts of solution varying from 0.25 to 0.50 ml. were applied to the spots in successive 10- to 20-μL. portions. Each portion was allowed to dry thoroughly before the next was applied. The spotted sheets were then placed in a glass chromatographic chamber, and the chromatograms were formed in the descending direction with a solvent system composed of 65% ethanol in water. The atmosphere of the chamber was allowed to equilibrate with the solvent for twenty-four hours prior to the introduction of the chromatograms. After drying, the chromatograms were sprayed with a saturated aqueous solution of cuprous chloride. As this reagent dried, greenish-yellow spots appeared where disulfiram was present. After spraying, these spots appeared dark purple under an ultraviolet lamp, but they did not fluoresce.

In this system, the R_f values obtained for disulfiram ranged from 0.70 to 0.74. Tailing was not objectionable, and the area of the disulfiram spot at the end of its travel was no more than two to three times the area of the original spot. A 100-mcg. quantity of disulfiram applied to the paper could be detected readily after formation of the chromatogram

Each of the concentrated extracts obtained from medium, mycelium, or carpophores was applied to the sheets in amounts of 0.25 and 0.50 ml. Each extract was also applied in a mixed spot with a 150-mcg. quantity of known disulfiram. Control spots of disulfiram[1] were also applied to each sheet.

Extraction Procedures.—The media of all cultures in a given series, i.e., those cultures of the same medium which had been inoculated on the same day, were combined after harvesting the mycelium. Each L. of medium was subjected to four successive extractions of five minutes duration with 200-ml. portions of chloroform. The four chloroform extracts were combined and allowed to evaporate spontaneously. The small amount of residue was redissolved in 5 ml. of chloroform per L. of medium and tested for presence of disulfiram.

The dried mycelium from each series of cultures was ground to a moderately coarse powder in a mortar, packed into a micropercolator, and extracted exhaustively with chloroform. This percolate was allowed to evaporate spontaneously, and the resulting residue was redissolved in 2 ml. of chloroform per Gm. of mycelium extracted. This concentrated extract was then tested for presence of disulfiram. No disulfiram was detected in either the medium or the mycelium of any of the cultures.

Carpophores of naturally-occurring *C. atramentarius* arising from the same mycelial source as those which had caused typical *Coprinus*-alcohol poisoning in R. Levin were investigated for the presence of disulfiram. A total fresh weight of 6.1 Kg. of mushrooms was processed by a method similar to that reported by Simandl and Franc (6). The mushrooms were allowed to autolyze for two days and were then reduced to a mash in a Waring Blendor. This mash was subjected to four successive extractions of five minutes duration with 300 ml. of carbon tetrachloride per Kg. of fresh mushrooms. The solids were separated from the solvent after each extraction by centrifugation. The combined carbon tetrachloride extracts were filtered and allowed to evaporate spontaneously. The fatty residue remaining was mixed with clean sand, packed into a glass percolator, and extracted exhaustively with anhydrous methanol. The methanol percolate was evaporated to dryness under vacuum at 30°. The slight gummy residue obtained in this way was redissolved in 10 ml. of anhydrous methanol per Kg. of fresh carpophores processed. No crystalline material was obtained. These extracts were purified by chromatographing in the manner previously described and eluting with anhydrous methanol the area which corresponded to the area where known disulfiram appeared on the same chromatograms. These purified extracts were then rechromatographed. No disulfiram was found in any of the carpophore extracts.

[1] Authentic disulfiram supplied through the courtesy of Ayerst Laboratories, New York, N. Y.

In order to ascertain if the extraction procedures which were employed would actually recover disulfiram, a quantity of that compound was added to a quantity of naturally-occurring carpophores of *C. atramentarius*. An 800-Gm. quantity of fresh mushrooms was reduced to a mash in a Waring Blendor, and 0.01% of disulfiram was added to one-half of the mash. After two days both portions of the mash were processed. The portion to which no disulfiram was added gave no test for disulfiram. The portion to which disulfiram was added was extracted, and the residue was dissolved in 10 ml. of anhydrous methanol. Fifty microliters of this concentrated extract were chromatographed and gave a color reaction with saturated cuprous chloride solution of greater intensity than 100 mcg. of known disulfiram. This indicated that the 10 ml. of extract contained over 20 mg. of disulfiram. The extraction procedure was therefore more than 50% efficient.

DISCUSSION

The quantities of mycelium produced by *C. atramentarius* when cultured on the seven media employed were extremely small. Medium No. 7, the horse dung extract medium, gave a slightly higher yield of the fungus than any of the other media tested. The small darkened areas of mycelial compaction which were observed when the fungus was grown on solid medium were similar to the structures described by C. H. Chow (8) as rudimentary carpophores; however, no true carpophores were produced.

The processes of extraction employed were shown to have an efficiency of at least 50%, and the chromatographic technique which was used could readily detect a 100-mcg. quantity of disulfiram. Consequently, the extracts of the culture media must have contained less than 0.2 mg. % of disulfiram or that compound would have been detected. Similarly, the mycelium extracts must have contained less than 80 mg. % of disulfiram, and the extracts of the naturally-occurring carpophores must have contained less than 0.4 mg. % of disulfiram (fresh weight basis). If disulfiram were present in the carpophores at a level just below that which was detectable by the procedures employed, approximately 125 Kg. of fresh mushrooms would have to be consumed in order to obtain the ordinary daily maintenance dose of 500 mg. of disulfiram (20). *C. atramentarius* arising from the same mycelial source as that investigated by the authors is known to possess disulfiram-like physiological activity when eaten in ordinary amounts. As the maximum possible level of disulfiram in the carpophores investigated by the authors is far below that which could exert physiological activity, it must be concluded that the disulfiram-like activity of these carpophores is due to some other agent present in the fungus.

The fact that disulfiram did not exist in the *C. atramentarius* investigated does not entirely rule out the possibility that a recoverable concentration of that compound did occur in the fungus investigated by Simandl and Franc (6). Such variation could be due to several factors. First, the positive identification of the black-spored Agarics is extremely difficult. One cannot be positive that the "*Coprinus atramentarius*" investigated by Simandl and Franc is identical with the organism investigated here. However, such a discrepancy is rather unlikely. Second, nutritional and environmental factors can alter the metabolism of organisms in such a way as to change, at least quantitatively, the products of that metabolism. Third, various genetic strains of *C. atramentarius* may exist which differ appreciably in their metabolism. The features upon which the taxonomy of the Coprini is based are chiefly morphological and anatomical in nature. Very little is known of the genetics of these organisms, and more than one genotype may well exist within a single phenotype.

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Interaction of Preservatives With Macromolecules IV*

Binding of Quaternary Ammonium Compounds by Nonionic Agents

By PATRICK P. DELUCA† and H. B. KOSTENBAUDER

Equilibrium dialysis studies utilizing a semipermeable nylon membrane indicate a high degree of association and accompanying inhibition of quaternary ammonium germicides such as cetylpyridinium chloride and benzalkonium chloride with nonionic surfactants such as Tween 80. Cetylpyridinium chloride was also found to bind to methylcellulose, but not to PVP or Polyox. Benzalkonium chloride was not bound to methylcellulose, PVP, or Polyox under the conditions of this study.

ALTHOUGH SEVERAL investigators have presented microbiological data indicating that nonionic surface-active agents can interfere with the activity of cationic germicides such as the quaternary ammonium compounds, textbooks and reference works generally do not emphasize the high degree of inactivation which can sometimes occur in these systems. It has been suggested that the observed inactivation is attributable to a preferential association or binding of the cationic agent with the nonionic surfactant (1), although no previous data have been published which would indicate the magnitude of this association. The present work was undertaken to obtain quantitative data for the degree of binding of several cationic agents by some typical nonionics and to compare the degree of binding with the degree of inactivation of the cationics.

Phospholipids such as lecithin have long been popular as neutralizing or inactivating media for quaternary ammonium compounds in germicidal testing (2–7). Quisno, Gibby, and Foter suggested the use of an inactivating medium consisting of lecithin and Tween 80, indicating that the lecithin was the primary inactivator with the Tween acting as a dispersing agent (8). In 1949 Gershenfeld and Stedman (9) reported their observations on the activity of several cationics, including cetylpyridinium chloride and cetyltrimethylammonium bromide, in the presence of varying concentrations of a nonionic surfactant, noting enhancement of activity at low surfactant concentration and inhibition at higher surfactant concentrations. Davies (10) reported that a polyethylene glycol cetostearyl ether was capable of neutralizing the bacteriostatic effect of cetyltrimethylammonium bromide and Ritter (11) indicated that Tween 80 was capable of neutralizing the bactericidal effect of cetylpyridinium chloride on *tubercle bacilli*. Barr and Tice (12) investigated seven quaternary ammonium compounds in the presence of 5% polyoxyethylene 20 sorbitan monostearate and found that only benzalkonium chloride was effective in a concentration of 0.1%. Wedderburn (13) indicated that benzalkonium chloride also was subject to inactivation by nonionics, including sucrose ester surfactants, under the conditions of her studies. Moore and Hardwick (1) presented microbiological data on combinations of quaternary ammonium compounds and nonionic surfactants and devoted considerable discussion to the relative effectiveness of such combinations. Other reports of inactivation are discussed in the recent review by Beckett and Robinson (14).

Studies cited above (1, 9) indicate that in very dilute solutions of the nonionic surfactants there is an enhancement of the effectiveness of the quaternary ammonium compound, but at higher concentrations of the surfactant the activity of the germicide is greatly diminished. This is typical of the behavior of many germicides in the presence of surface-active agents (1, 15, 16).

In the present study two typical quaternary ammonium compounds, cetylpyridinium chloride and benzalkonium chloride, were selected to illustrate the relative magnitude of any interaction which might occur between these agents and nonionics such as Tween 80, methylcellulose, polyvinylpyrrolidone, and high molecular weight ethylene oxide polymers. The particular quaternary ammonium compounds used in this study were selected principally because both agents show absorption in the ultraviolet and thus can readily be determined spectrophotometrically.

EXPERIMENTAL

Reagents.—Cetylpyridinium chloride (1-hexadecylpyridinium chloride);[1] benzalkonium chloride U. S. P. (a mixture of alkyldimethylbenzylammonium chlorides in which the alkyls range from C_8H_{17} to $C_{18}H_{37}$);[2] cetyldimethylbenzylammonium

* Received August 21, 1959, from the School of Pharmacy, Temple University, Philadelphia, Pa. Revised January 1960.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
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[1] Ceepryn Chloride, The Wm. S. Merrell Co., Cincinnati, Ohio.
[2] Zephiran Chloride, Winthrop-Stearns Inc., New York, N. Y.

chloride;[3] Tween 80,[4] a commercial sample and also a sample passed through an ion exchange column; methylcellulose, 15 c. p. s.;[5] polyvinylpyrrolidone;[6] Polyox WSR-35.[7]

To assure that any observed inactivation of quaternary ammonium compounds by Tween 80 would not be attributable to the presence of unesterified oleic acid in the Tween sample, the Tween solution was passed through a mixed-bed ion exchange column[8] to remove traces of oleic acid which might be present. Quantitative removal of oleic acid was established by adding known amounts of free oleic acid to Tween 80 samples and determining oleic acid content by potentiometric titration with approximately 0.1 *N* NaOH before and after passage through the ion exchange resin. Subsequent studies indicated that the contribution of any traces of oleic acid in the commercial sample of Tween 80 to the inactivation of quaternary ammonium compounds was probably insignificant.

Before the polyvinylpyrrolidone was employed in the dialysis studies, the sample was extracted with ether in a Soxhlet extractor for forty-eight hours.

Conductivity Measurements.—To establish approximate critical micelle concentrations for the samples of cetylpyridinium chloride and cetyldimethylbenzylammonium chloride used in this study, conductivity measurements were obtained for aqueous solutions at room temperature (23–25°). An Industrial Instruments model RC M15 conductivity bridge and a dipping type conductivity cell with a cell constant of approximately 1.00 $cm.^{-1}$ were employed. The water used was that used throughout the study and had a specific conductance of approximately 2×10^{-1} ohm^{-1}.

Dialysis Studies.—Dialysis membranes employed in the studies involving Tween 80 were nylon membranes as described previously by Patel and Kostenbauder (18). These membranes were previously shown to be impermeable to Tween 80 (18). For dialysis studies involving methylcellulose, polyvinylpyrrolidone, and Polyox, Visking cellulose casings were employed. The general procedure for these studies consisted of placing inside the dialysis bag 20 ml. of a solution containing the quaternary ammonium compound and, after tightly knotting the open end, placing the bag into a 125-ml. bottle containing 40 ml. of Tween or other polymer solution. A polyethylene film and a screw cap were employed as the closure, and the bottles were rotated at 9 r. p. m. in a constant temperature bath at 30°. As might be expected for the long chain quaternary ammonium compounds, equilibration times of five to seven days were necessary for cetylpyridinium chloride solutions and two to three days for benzalkonium chloride solutions. The presence of Tween 80, however, reduced the equilibration time for cetylpyridinium chloride to one to two days.

After equilibration, aliquots were removed from both sides of the membrane and concentrations of quaternary ammonium compounds were determined spectrophotometrically at a wavelength of 259 mμ for cetylpyridinium chloride and 261.5 mμ for benzalkonium chloride, using a Beckman DU spectrophotometer. Any interference due to the macromolecule was eliminated by using the appropriate concentration in the reference cell.

Microbiological Studies.—Observations were made on the influence of several nonionic surface-active agents on the bacteriostatic activity of cetylpyridinium chloride and benzalkonium chloride, using *Aerobacter aerogenes*,[9] a Gram-negative organism. The methods and culture medium previously described by Pisano and Kostenbauder (19) were employed. Approximate inhibitory concentrations for cetylpyridinium chloride and benzalkonium chloride in the presence of Tween 80, Triton X-100,[10] and Pluronic L62,[11] were obtained through visual observation of samples each day for a period of two weeks.

RESULTS

Critical Micelle Concentration for Cationics.—Approximate critical micelle concentrations for cetylpyridinium chloride and cetyldimethylbenzylammonium chloride (CDBAC) were obtained from the conductivity data illustrated in Fig. 1. The critical micelle concentration for cetylpyridinium chloride was found to be approximately 1.0×10^{-3} *M*, while that for the CDBAC was found to be approximately 1.0×10^{-4} *M*.

Tween 80.—Cetylpyridinium chloride was found to interact to an extremely high degree with Tween 80 when the cationic was employed in concentrations comparable to those which might be encountered in product formulations. Figures 2 and 3 illustrate typical adsorption isotherms for this system, and in Fig. 4 these data are presented in a manner which indicates that in an aqueous system containing Tween 80 and cetylpyridinium chloride, the ratio of total to free cetylpyridinium chloride is primarily a function of the concentration of Tween 80. As illustrated in a previous publication (19), the ratio of total to free germicide can often be employed to predict required preservative concentrations in the presence of the Tween. Figure 4 indicates that at a concentration of 1% Tween 80, approximately 95% of the cetylpyridinium chloride present is bound to the Tween and thus inactivated. Even at a concentration of 0.1% Tween, the data suggest that approximately 60% of the total cetylpyridinium chloride would be inactivated. The dialysis studies indicated no significant difference in binding of cetylpyridinium chloride by a commercial sample of Tween 80 and a sample passed through an ion exchange resin to remove any oleic acid present.

[3] Sample obtained through the courtesy of Dr. George D. Wessinger, Sterling-Winthrop Research Institute, Rensselaer, N. Y.

[4] Tween 80 is polyoxyethylene 20 sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

[5] Methocel, 15 c. p. s., the Dow Chemical Co., Midland, Mich.

[6] Plasdone, Antara Chemicals Division of General Aniline and Film Corp., New York, N. Y.

[7] Polyox WSR-35 is a poly(ethylene oxide) of exceptionally high molecular weight (17), Union Carbide Chemicals Co., New York, N. Y.

[8] The ion exchange column contained a mixture of the strongly basic quaternary amine ion exchange resin Amberlite IRA-400, previously treated with NaOH solution, and the cation exchange resin Amberlite IR-120, previously treated with HCl solution.

[9] ATCC No. 8308.

[10] Triton X-100 is an alkylaryl polyether alcohol, Rohm and Haas Co., Philadelphia, Pa.

[11] Pluronic L62 is a polyoxyethylene-polyoxypropylene surfactant, Wyandotte Chemicals Corp., Wyandotte, Mich.

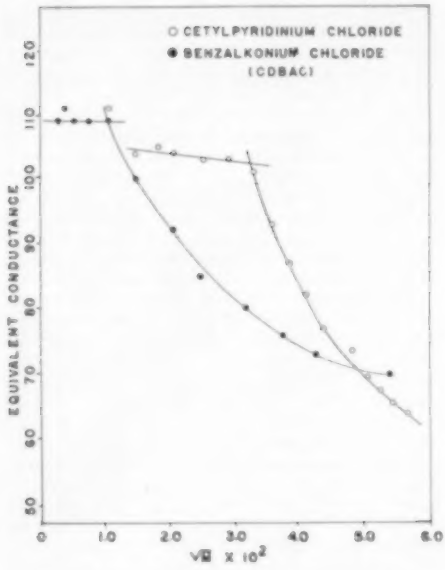

Fig. 1.—Determination of critical micelle concentration for cetylpyridinium chloride and cetyldimethylbenzylammonium chloride through conductivity measurements, 23–25°. Critical micelle concentrations were approximately 1.0×10^{-3} *M* for cetylpyridinium chloride and 1.0×10^{-4} *M* for cetyldimethylbenzylammonium chloride.

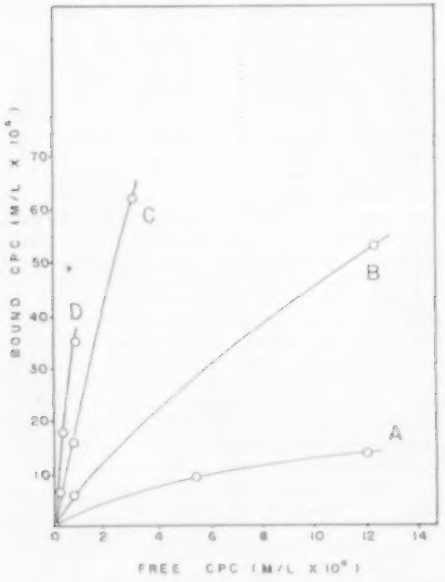

Fig. 2.—Adsorption isotherms for binding of cetylpyridinium chloride by Tween 80 in aqueous solution at 30°. *A*, 0.1%; *B*, 0.5%; *C*, 1.0%; *D*, 2.5% TW 80.

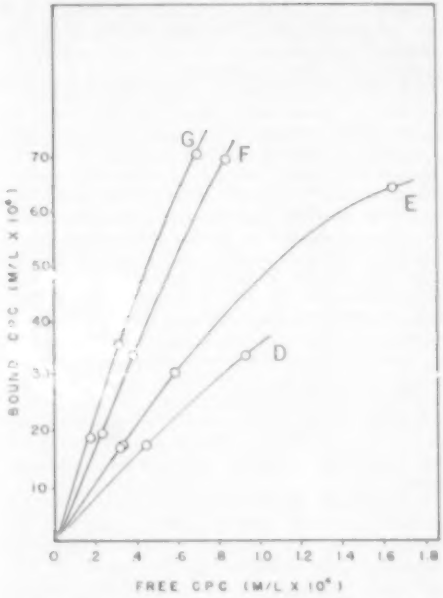

Fig. 3.—Adsorption isotherms for binding of cetylpyridinium chloride by Tween 80 in aqueous solution at 30°. *D*, 2.5%; *E*, 5.0%; *F*, 7.5%; *G*, 10.0% TW 80.

The marked scatter of points in Fig. 4 is partly a result of magnification of experimental error due to the pronounced binding which occurs. Calculation of r, the ratio of total to free preservative, in some cases involves dividing the total concentration by a number smaller by a factor of approximately 100; thus, a small error in either total or free concentration is greatly magnified in calculation of the r value. Despite the inherent inaccuracy of such treatment of data, the convenience of a graph such as that presented in Fig. 4 justifies its presentation.

Data for the binding of benzalkonium by Tween 80 are illustrated in Fig. 5. While the binding of benzalkonium chloride is considerably less than that exhibited by cetylpyridinium chloride, it nevertheless is sufficient to cause inactivation. At a concentration of 1% Tween 80, approximately 50% of the benzalkonium chloride present is bound to the Tween. There appeared to be no significant difference in the degree of binding exhibited by benzalkonium chloride U. S. P., which is described as consisting of alkyls in the range $C_8 - C_{18}$ (20), and the binding shown by a pure sample with a C_{16} chain, i. e., CDBAC.

Figure 6 represents adsorption isotherms for the interaction of cetylpyridinium chloride and CDBAC with a very dilute solution of Tween 80. This study was included to permit observations on the degree of binding in the presence of free quaternary concentrations both above and below the normal critical micelle concentration for the quaternary compound. These isotherms differ markedly from those obtained in higher concentrations of Tween

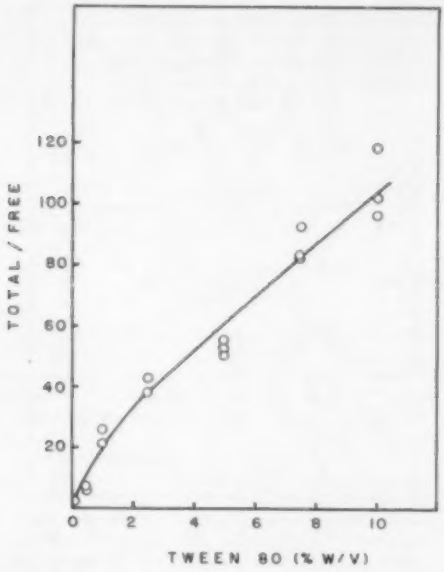

Fig. 4.—The data illustrated in Figs. 2 and 3 plotted to give the ratio, r, of total to free cetylpyridinium chloride as a function of the concentration of Tween 80.

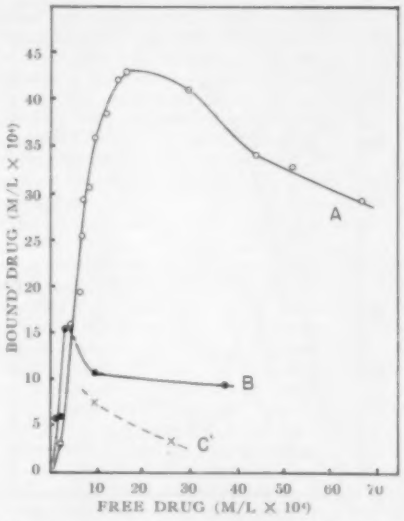

Fig. 6.—Adsorption isotherms for binding of cetylpyridinium chloride and cetyldimethylbenzylammonium chloride in 0.2% Tween 80 at 30°. These curves illustrate the degree of binding occurring at concentrations of the quaternary ammonium compounds both above and below their normal critical micelle concentrations. *A*, cetylpyridinium Cl; *B*, benzalkonium Cl(CDBAC); *C*, cetylpyridinium Cl, 0.05 *M* NaCl. Curve *C* illustrates the influence of electrolyte on the degree of interaction.

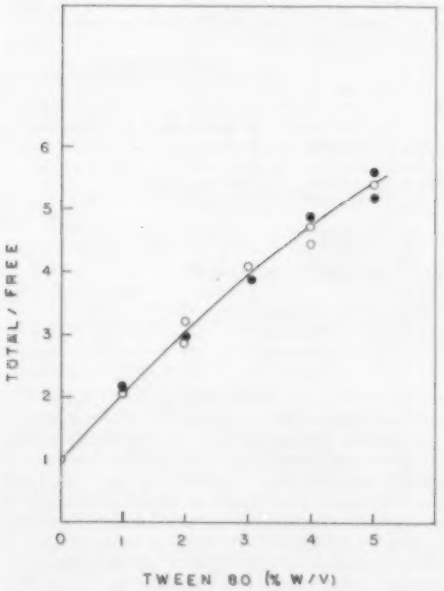

Fig. 5.—The ratio, r, of total to free benzalkonium chloride as a function of Tween 80 concentration at 30°. Total benzalkonium chloride concentration 0.025–0.10%. ○, benzalkonium chloride U. S. P.; ●, cetyldimethylbenzylammonium chloride.

80, showing a maximum in the adsorption isotherm in the region of the critical micelle concentration.

Methylcellulose.—Figure 7 shows the interaction of cetylpyridinium chloride with methylcellulose. The binding is highly dependent on the cetylpyridinium chloride concentration, passing through a maximum in the region of the normal critical micelle concentration for cetylpyridinium chloride. Benzalkonium chloride was found to exhibit no detectable binding to methylcellulose up to a concentration of 2% methylcellulose and 0.125% cationic.

Figure 8 shows the influence of pH and electrolyte on the interaction of cetylpyridinium chloride with methylcellulose. The binding appears to be increased somewhat in the presence of strong base, but is suppressed by 0.05 *M* NaCl and almost eliminated by 0.05 *M* HCl.

Polyvinylpyrrolidone.—Neither cetylpyridinium chloride nor benzalkonium chloride was found to interact significantly with polyvinylpyrrolidone at a concentration of 2% polyvinylpyrrolidone and 0.08 % quaternary ammonium compounds.

Polyox.—Neither cetylpyridinium chloride nor benzalkonium chloride was found to interact significantly with this polymer at concentrations of 0.2% Polyox and 0.07% quaternary ammonium compound.

Microbiological Studies.—Concentrations of cetylpyridinium chloride and benzalkonium chloride required to inhibit growth of *Aerobacter aerogenes* in the presence of several nonionic surfactants are

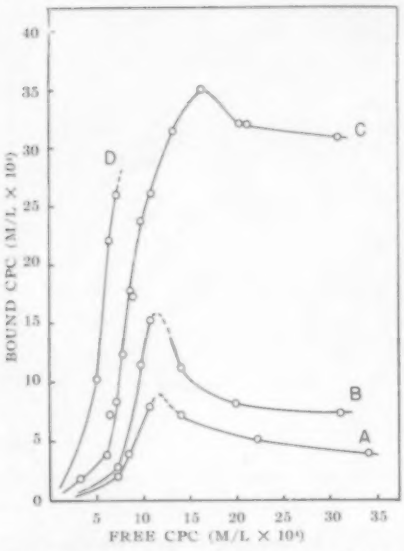

Fig. 7.—Binding of cetylpyridinium chloride by methylcellulose at 30°. *A*, 0.05%; *B*, 0.1%; *C*, 0.5%; *D*, 1.0% methylcellulose.

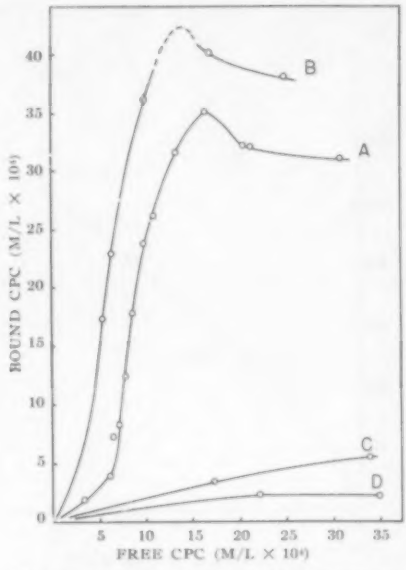

Fig. 8.—Influence of pH and electrolyte on the interaction of cetylpyridinium chloride with 0.5% methylcellulose in aqueous solution at 30°. *A*, distilled water; *B*, 0.003 *M* NaOH; *C*, 0.05 *M* NaCl; *D*, 0.05 *M* HCl.

presented in Table I. These studies were made with polymer solutions which were passed through ion exchange resins to remove any fatty acid or electrolyte present and also with some untreated samples of Tween 80. No attempt was made to determine precise inhibitory concentrations, but the increased concentrations of quaternary ammonium compound indicate a somewhat higher degree of inactivation than would be predicted from a consideration of Figs. 4 and 5.

As would be predicted from the binding studies, benzalkonium chloride was found to be inhibited to a lesser degree than was the cetylpyridinium chloride. This observation is in agreement with that of Barr and Tice (12), who found that in the presence of Tween 60 benzalkonium chloride was effective in a concentration of 0.1% while cetylpyridinium chloride was not

The results obtained with Pluronic L62 and Triton X-100 indicate that these agents show an ability to inactivate the quaternary ammonium compounds quite similar to that exhibited by Tween 80.

Table I.—Influence of Several Nonionic Surfactants on Concentrations of Cationic Required to Inhibit *Aerobacter aerogenes*

Nonionic	Inhibitory Concentration: Cetylpyridinium Cl	Inhibitory Concentration: Benzalkonium Cl
0	1–100,000 to 1–250,000	No growth at 1–100,000
0.5% Tween 80[a]	1–2,500 to 1–5,000	....
2.0% Tween 80[a]	1–250 to 1–500	....
3.0% Tween 80[b]	1–100 to 1–250	1–500 to 1–1,000
3.0% Triton X-100[b]	1–100 to 1–250	....
3.0% Pluronic L62[b]	1–500 to 1–1,000	....

[a] Commercial sample. [b] Treated with ion exchange resin before use.

DISCUSSION

Tween 80.—The formation of mixed micelles of quaternary ammonium compound and nonionic surfactant has been suggested as a possible mechanism for the association with molecules such as Tween 80 (1). For such an interaction, the degree of binding would be expected to increase with increasing length of the hydrocarbon chain of the cationic, at least so long as the concentration of the free cationic did not exceed the normal critical micelle concentration. The critical micelle concentrations for the cetylpyridinium chloride and CDBAC used in these studies were found to be approximately 1.0×10^{-3} *M* and 1.0×10^{-4} *M*, respectively. Only in the concentrations of Tween below 0.5% were free cetylpyridinium concentrations of this magnitude approached, although in all cases the concentration of free benzalkonium was in excess of the critical micelle concentration. It was at first suspected that the marked difference in degree of binding of cetylpyridinium chloride and benzalkonium chloride by the Tween might be a result of heterogeneity in the composition of the alkyl of the benzalkonium chloride, since the U. S. P. specifies C_8-C_{18} for this compound. However, subsequent binding studies using a pure sample of cetyldimethylbenzylammonium chloride (CDBAC) resulted in data which exactly duplicated the degree of binding exhibited by the U. S. P. product.

If the interaction between the quaternary ammonium compound and the Tween can be visualized as an equilibrium between mixed micelles of quaternary ammonium-Tween and individual quaternary ammonium ion and halide ion

$$aQ^+ + bCl^- + cTw \rightleftarrows Q^+_aCl^-_bTw_c$$

the interaction would be expected to be dependent on the concentration[12] of the long chain cation according to the law of mass action.[13]

$$K' = \frac{(Q^+_aCl^-_bTw_c)}{(Q^+)^a(Cl^-)^b(Tw)^c} \qquad \text{(Eq. 1)}$$

In Eq. 1, K' is an equilibrium constant, (Tw) is the free Tween concentration, (Q^+) is the concentration of individual long chain cation, (Cl^-) is the concentration of the individual halide ion, and $(Q^+_aCl^-_bTw_c)$ is the concentration of the mixed micelle or complex.

Several investigators have observed that in an aqueous solution of an ionic surfactant the concentration of the individual long chain ion passes through a maximum as the total concentration of surfactant in the solution is increased. This maximum occurs in the concentration range at which micelle formation is observed. The existence of the maximum can be shown by the application of the law of mass action to micelle formation, the most extensive treatments being the work of Mysels (21) and of Sexsmith and White (22, 23). For the system $aQ^+ + bX^- \rightleftarrows Q^+_aX^-_b$ where

$$K'' = \frac{(Q^+_aX^-_b)}{(Q^+)^a(X^-)^b} \qquad \text{(Eq. 2)}$$

Sexsmith and White, by solving for (Q^+) as a function of total quaternary concentration, (C), and setting $d(Q^+)/d(C) = 0$, demonstrated that for several model quaternary ammonium surfactants the concentration of individual quaternary ammonium ion, (Q^+), passes through a maximum if $a>b\geqq 2$. The concentration of the individual halide ion, (X^-), and the ion product of quaternary ammonium ion and halide ion, however, continue to increase as the total quaternary ammonium concentration is increased.

The existence of this maximum in individual quaternary ammonium ion concentration provides a probable explanation for the observed differences in binding affinities of cetylpyridinium chloride and CDBAC. If the concentration of individual quaternary ammonium ion parallels the total free quaternary ammonium compound concentration until micelles begin to appear, it might be expected that the approximately tenfold difference in critical micelle concentration for the cetylpyridinium chloride and CDBAC would result in a maximum attainable concentration of individual quaternary ammonium ion greater by approximately a factor of 10 for the cetylpyridinium chloride solutions. Occurrence of a maximum in individual quaternary ammonium ion at extremely low concentrations of CDBAC suggests that the concentration of single ions of cetyldimethylbenzylammonium never approaches the level encountered in cetylpyridinium solutions. For an interaction between a quaternary ammonium compound and Tween 80, such as that illustrated in Eq. 1, a tenfold difference in quaternary ion concentration would be expected to exert a considerable influence on the degree of binding occurring.

If the proposed mechanism is operative, it should be possible to demonstrate maxima in the adsorption isotherms for the interaction of cetylpyridinium chloride and CDBAC with Tween 80. To this end, the binding data illustrated in Fig. 6 were obtained, using a very dilute solution of Tween 80. The existence and relative positions of the maxima in the adsorption isotherms are very clearly illustrated. These data indicate that the interaction is dependent on the concentration of the single quaternary ammonium ion and could not be attributed solely to the adsorption of ion pairs or micelles, since both the product of (Q^+) (Cl^-) and the concentration of micelles are known to increase steadily as the total quaternary concentration increases (23). The decrease in the degree of interaction at higher quaternary concentrations is most satisfactorily explained thermodynamically on the basis of a decrease in concentration of the individual quaternary ion.

Observations on the interaction of Tween 80 and cetylpyridinium chloride in the presence of 0.05 M NaCl are indicated by the broken line in Fig. 6. These points suggest a shift of the maximum in the adsorption isotherm to lower concentrations, and suggest that the presence of electrolyte might be a rather significant factor in determining the degree of binding.

The binding of organic electrolytes by nonionic surfactants is not limited to quaternary ammonium compounds; drugs such as chlorpromazine, promethazine, and tetracaine hydrochlorides are also bound, as are dyes and some anionic detergents (24).

Methylcellulose.—Available data for the binding of cations to cellulose derivatives pertain principally to those agents used in dyeing textiles. Numerous theories have been presented to account for the binding of dyes and metal ions to cellulose, some of which include: (*a*) ionic binding to residual carboxyls or to acidic hydroxyls by ion exchange, (*b*) ion-dipole interactions of cations with ethers or hydroxyls, (*c*) dipole-dipole interactions between hydroxyls of cellulose and polar groups of the dye molecule, (*d*) interactions between hydrocarbon portions of the adsorbate and the linear cellulose molecules.

More recent discussions seem to indicate that more than one mechanism is involved in most cases of adsorption to cellulose (22). Until recently the importance of the hydrophobic interaction was not fully recognized. It now appears to have been established that for a dye to have maximum affinity for a cellulose substrate the dye should be a long, planar molecule which can attain close approach to the linear cellulose chain. Where this condition is met there is an extremely high degree of stability for the dye-cellulose combination; the stability being attributed to interaction of the hydrophobic groups (25).

[12] Here the assumption is made that concentrations may be substituted for activities.

[13] The interaction would, of course, also be dependent on the concentration of the Tween and the halide ion, but these considerations are not vital to the discussion which follows.

Figure 7 illustrates the binding of cetylpyridinium chloride by methylcellulose as a function of methylcellulose and cetylpyridinium chloride concentration. The interaction shows a high dependency on cetylpyridinium concentration and the initial portion of the curve, showing a rapidly increasing slope, is similar in appearance to the binding of phenol by PVP and polyethylene glycols (26) and to the binding of phenol (27, 28) and organic ions (29, 30, 31) by proteins. Curves such as this are sometimes considered to result when adsorption of a critical quantity of drug results in a change in configuration of tightly coiled polymer molecules to make additional binding sites available. A curve of this nature, which seemingly indicates limitless binding capacity, is also similar to multilayer adsorption isotherms, and in this case might be explained on the basis of possible tail-to-tail adsorption of quaternary after the initial adsorption of a critical number of ions. A mechanism of this nature has recently been demonstrated for the binding of cationic dyes and surfactants by cotton, viscose rayon, and oxycellulose (22, 23, 32, 33). These workers demonstrated that the ion exchange process involved residual carboxyls in the cellulose.

A recent report by Fishman and Miller (34) indicates that starch also interacts with quaternary ammonium compounds, and from the data presented the mechanism for the interaction would appear to be quite similar to that which occurs with cellulose and methylcellulose.

Data presented in Fig. 8 illustrate the effect of pH and electrolyte on the degree of binding, and the results are indicative of an ion exchange mechanism as the initial step in the interaction. The interaction is apparently enhanced by dilute alkali, greatly diminished by 0.05 *M* NaCl, and almost entirely eliminated by 0.05 *M* HCl.

The maximum which appears in the adsorption isotherm occurs in the neighborhood of the critical micelle concentration for cetylpyridinium chloride and is typical of adsorption isotherms described by Sexsmith, *et al.* (22, 23, 32, 33), for the interaction of quaternary ammonium compounds with cellulose. Sexsmith, *et al.*, demonstrated that the existence of the maximum can be attributed to the occurrence of a maximum in the concentration of individual quaternary ion and the resultant maximum in the ion exchange adsorption (22, 23).

Failure of benzalkonium chloride to interact with methylcellulose can be attributed to the relatively low concentration of individual quaternary ion which can occur in these solutions. Failure of Polyox and PVP to interact with the quaternary ammonium compounds can probably be attributed to the nonionic and nonionogenic nature of these polymers.

Microbiological Observations.—The estimation of inhibitory concentrations of cationic agents in the presence of the nonionics indicated an even higher degree of inactivation of the cationic than would be predicted from the equilibrium dialysis studies. This discrepancy can probably be attributed to the presence of approximately 0.1 *M* total electrolyte in the culture medium. Addition of electrolyte is known to lower the critical micelle concentration for quaternary ammonium compounds (35), and at concentrations below the critical micelle concentration the presence of electrolyte would also be expected to enhance the interaction between the cationic and the Tween. While an exact correlation between binding data and microbiological data would apparently require knowledge of the interaction as a function of electrolyte concentration, the dialysis studies presented here do verify the existence of a remarkably high degree of interaction for some quaternary ammonium germicides with nonionics such as Tween 80 and methylcellulose, and illustrate the perils of making the general assumption that nonionic agents are compatible with cationic and anionic drugs.

SUMMARY

1. Equilibrium dialysis studies indicate an extremely high degree of association and accompanying inactivation of quaternary ammonium germicides in the presence of nonionic surface-active agents. In 1 per cent aqueous solutions of Tween 80 approximately 95 per cent of the total cetylpyridinium chloride or 50 per cent of the total benzalkonium chloride present would be bound to the Tween and thus inactivated. In 0.1 per cent Tween 80 solution approximately 60 per cent of the cetylpyridinium chloride would be bound.

2. Methylcellulose was found to interact significantly with cetylpyridinium chloride, but not with benzalkonium chloride. Polyvinylpyrrolidone and Polyox were not found to interact with these cationics.

3. These studies indicate quite clearly that it is not justifiable to assume that nonionic agents are always compatible with cationic and anionic drugs.

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Urinary Excretion Kinetics for Evaluation of Drug Absorption III*

Method for Calculation of Absorption Rate and Application to Tetracycline Absorption in Humans

By EINO NELSON

A method was developed to calculate drug absorption rate from urinary excretion rate measurements and applied to study tetracycline absorption in humans after oral ingestion of 25, 50, 100, and 200-mg. doses. Calculation of absorption rate indicated that this drug was absorbed at a rate of about 100 to 140 mg./hr., one hour after 200-mg. doses, and at about 20 to 30 mg./hr., one hour later. Rates at these times decreased when the dose was reduced. Wide individual variation in absorption rate was observed among members of the test panel.

THERE ARE relatively few methods available that can be applied to study drug absorption quantitatively in the intact animal after drug ingestion orally. The method of Dominguez (1) may be applied, but its use requires frequent blood samples at short intervals of time after drug ingestion. The intubation method of Nicholson and Chornock (2) allows accurate measurement, but is an uncomfortable procedure to the test subject. The present work describes a method that is based on data collected from urinary excretion measurements. The method is applicable to all routes of drug administration, but is applied here to the oral route to study the mechanism of tetracycline's absorption in humans.

THEORY AND DEVELOPMENT OF THE METHOD FOR ABSORPTION RATE CALCULATION

The method is based on writing a material balance accounting for drug at absorption sites, in the body, and eliminated drug in excreted urine or removed by other processes. Several assumptions must be made and conditions met in order to make the method experimentally and mathematically tractable. These conditions and assumptions are: (*a*) A significant amount of drug must be excreted unchanged in the urine. While there can be no arbitrarily set lower limit on the percentage eliminated by this route, it is desirable that this percentage be at least of the order of 30. Error in rate calculation increases markedly with error in percentage when the latter is a low value. (*b*) It is necessary to assume that drug in circulation is in equilibrium with other fluids of distribution at all times even though there is always a delay in the attainment of this equilibrium. (*c*) After this equilibrium is reached, the drug must disappear from the blood stream by an apparent first-order process which is the usual case.

At any time the dose of a drug taken can be accounted for by the following expression

$$A_d = A_b + A'_e + A_t \qquad \text{(Eq. 1)}$$

In Eq. 1, A_d is the dose, A_b is the amount in the body in blood and other fluids of distribution, A'_e is the amount eliminated by urinary excretion and other processes, and A_t is the amount of drug remaining at the absorption sites. It follows on differentiation of Eq. 1 with respect to time and rearrangement that

$$-dA_t/dt = dA_b/dt + dA'_e/dt \qquad \text{(Eq. 2)}$$

* Received September 19, 1958, from the School of Pharmacy, University of California Medical Center, San Francisco 22.
Supported in part by a Grant-in-aid from the Squibb Institute for Therapeutic Research, New Brunswick, N. J.

In Eq. 2, t is time, dA_t/dt is the rate of disappearance of drug from the absorption sites, dA_b/dt is the rate of change of amount of drug in the body, and dA'_e/dt the rate of elimination from the body.

The rate of elimination may be deduced from excretion data. This rate is equal to the sum of the urinary excretion rate, dA_e/dt and the rate of elimination by other processes, dA''_e/dt, i. e.

$$dA'_e/dt = dA_e/dt + dA''_e/dt \qquad \text{(Eq. 3)}$$

As a consequence of the considerations discussed by Dominguez and Pomerene (3) and later by Hough (4), a simple relationship exists between the two righthand terms of Eq. 3. These terms are, respectively, equal to k_1A_b and k_2A_b where k_1 and k_2 are the rate constants in reciprocal hours for each process. Hence

$$dA'_e/dt = A_b(k_1 + k_2) \qquad \text{(Eq. 4)}$$

It helps to define, $K = k_1 + k_2$, where K is the rate constant that describes the rate of removal by the combined processes. Also in accord with the considerations discussed by Hough (4), the rate of excretion in the urine may be described by the following

$$dA_e/dt = KfA_b \qquad \text{(Eq. 5)}$$

where f is the fraction of drug reaching circulation that is eliminated unchanged in the urine. Solving Eq. 5 for A_b and substituting in Eq. 4 yields

$$dA'_e/dt = (1/f)(dA_e/dt) \qquad \text{(Eq. 6)}$$

which when substituted in Eq. 2 gives

$$-dA_t/dt = dA_b/dt + (1/f)(dA_e/dt) \qquad \text{(Eq. 7)}$$

The term, dA_b/dt, in Eqs. 2 and 7 may be found from Eq. 5. Taking the derivative of Eq. 5 and solving for dA_b/dt obtained by this process and substituting for its value in Eq. 7 yields

$$-dA_t/dt = (1/Kf)(d^2A_e/dt^2) + (1/f)(dA_e/dt) \qquad \text{(Eq. 8)}$$

Since absorption rate is the same as disappearance rate only with opposite sign, Eq. 8 may be stated as

$$\text{Absorption rate} = (1/Kf)(d^2A_e/dt^2) + (1/f)(dA_e/dt) \qquad \text{(Eq. 9)}$$

The rate terms in Eq. 9 may be obtained graphically or by use of polynomials fitted to excretion data as will be described later. The value of K for a given drug may be found from either the literature or from the linear portion of a plot of the logarithm of excretion rate *vs.* time, where excretion rate may be either the graphically determined values or values obtained from the amount excreted in a given time divided by the time interval over which the sample was collected. The value of K is easily found from such a plot by noting the time necessary for a 50% decrease in rate and making use of the relationship, $K = 0.693/t_{1/2}$, where $t_{1/2}$ is the half life noted.

Calculation of rate of absorption by Eq. 9 is independent of the size of the dose given or the percentage of this dose absorbed. In fact, if for example, the same drug is given in several different forms such as different salts, calculation of absorption rate gives the absorbability of each form.

Collection of Data.—Collection of excretion data from a given drug for use in the application of Eq. 9 requires some consideration of excretion velocity in order to obtain meaningful results. When the cumulative amount excreted *vs.* time curve is constructed, based on data obtained after administration of drug by other than intravenous means, it always shows an inflection point. To characterize properly the excretion curve, at least two urine collections should be made before the appearance of this inflection point and at least two afterwards. Spacing of urine collections will depend on the rapidity with which the drug is absorbed and excreted. The experimental data in this report indicate collection period spacing to some extent.

Comparison with Other Methods of Calculating Absorption Rate.—The only other methods available to calculate absorption rate are those in which unabsorbed material is recovered from experimental animals (2), or the method applied by Dominguez (1) to creatinine. The method of Dominguez (1) requires the assumption that drug in blood and other fluids of distribution is in equilibrium at all times as is the case in the method presented here. However, the present method does not require information on the total volume of distribution of drug which is required in the Dominguez method. The determination of this quantity is subject to some error.

The approximate method for calculating absorption rate presented here is generally applicable under the conditions stated and has been presented in some detail. Use of the method does not depend on the site of drug administration, although it is applied here to absorption from the gastrointestinal tract.

EXPERIMENTAL PROCEDURE

Test Subjects.—The test panel consisted of five adults in apparent good health. Their ages and weights are given in the first entry of Table I.

Dosage Forms.—Tetracycline hydrochloride was given in the form of particles with an average diameter of 100 μ contained in hard gelatin capsules mixed with 200 mg. sodium bicarbonate. The particles and dosage forms were prepared as previously described (5). The tetracycline hydrochloride was of commercial purity without additives. Four sets of dosage forms, each set containing 25, 50, 100, and 200 mg. tetracycline hydrochloride, respectively, were used in the tests. The salt and dosage form used was to insure that absorption would not be rate-limited by solution rate (5).

Conduction of Tests and Assay.—The drug was taken on fasting stomachs in the morning under conditions which have been previously described (5). The dosage for a given subject on a given test day was randomly selected.

Following ingestion, subjects collected urine specimens at one, two, three, and four hours, and aliquots of these, as well as aliquots of preingestion specimens, were assayed microbiologically for tetracycline hydrochloride activity by the cup plate method (6).[1] The tetracycline hydrochloride standard for comparison was prepared from the same batch used to prepare the dosage forms.

[1] The author is indebted to Mrs. Yoko Yuzuriha for conduction of these assays.

TABLE I.—CUMULATIVE MG. TETRACYCLINE HYDROCHLORIDE AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS[a]

Subject[b]	mg. Excreted				ml. Urine			
	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0
25-mg. Dose Tetracycline HCl								
E (39–77)	0.8	1.7	2.7	3.9	340	400	500	870
S (33–57)	0.7	2.1	3.4	4.2	80	150	220	260
N (21–77)	0.2	1.7	4.0	5.1	260	460	530	600
C (29–84)	0.1	0.5	0.8	1.0	70	170	230	280
J (26–107)	0.2	0.6	1.2	1.8	60	130	190	260
Mean	0.4	1.3	2.4	3.2	162	262	334	454
50-mg. Dose Tetracycline HCl								
E	1.8	5.1	7.8	9.8	110	190	460	645
S	1.3	3.4	5.9	7.8	90	200	350	460
N	0.4	3.9	8.1	10.5	110	210	270	320
C	0	1.1	5.4	9.3	80	140	180	210
J	1.4	5.7	8.9	11.5	80	170	250	310
Mean	1.0	3.8	7.2	9.8	94	182	302	389
100-mg. Dose Tetracycline HCl								
E	0	1.8	6.3	9.9	210	360	680	1140
S	1.8	6.2	9.8	13.2	70	150	240	450
N	1.0	6.0	13.0	18.6	40	140	330	430
C	1.9	11.9	17.9	26.6	60	110	180	250
J	1.9	9.7	15.2	22.4	210	400	540	710
Mean	1.3	7.1	12.4	18.1	118	232	394	596
200-mg. Dose Tetracycline HCl								
E	2.8	10.3	18.7	24.7	250	570	670	790
S	2.4	10.2	21.1	29.5	270	510	590	790
N	3.8	16.0	25.0	31.0	70	150	190	230
C	0.6	7.3	15.8	27.5	110	240	350	410
J	1.8	6.8	13.0	16.9	40	80	140	230
Mean	2.3	10.1	18.7	25.9	148	310	388	490

[a] All values in terms of the activity of tetracycline hydrochloride.
[b] Quantities in parentheses are subject's age followed by his weight in Kg.

RESULTS AND DISCUSSION

Tetracycline Excretion.—Table I summarizes excretion data with pertinent related information. In almost all cases shown the excretion values listed were from a collection at the indicated times. In the few cases where urine collections could not be made exactly on the hour, individual plots of cumulative amounts excreted *vs.* time curves were constructed and excretion values for entry in Table I determined by interpolation. The mean values on Table I are plotted on Fig. 1.

Fitting Experimental Data to Polynomials in Powers of Time.—The mean values for amounts of tetracycline excreted from the several dosage schemes shown in Table I were fitted to polynomials in powers of time of the form

$$Ae = at + bt^3 + ct^5 + dt^4 \quad \text{(Eq. 10)}$$

The individual data from 200-mg. doses of the drug were also fitted to equations of this form. The values of the coefficients of Eq. 10 are given in Table II for the combined excretion from various doses. Table III lists the coefficients of Eq. 10 as obtained by using the individual data of test subjects after they ingested 200-mg. doses.

The first and second derivatives of Eq. 10 with coefficients evaluated were used to supply these respective terms needed in Eq. 9. After dividing the value of the second derivative by K, it was then possible to described absorption rate in terms of its product with f. This quantity was then calculated at one and two hours for each of the several doses.

Illustrative Example.—Fitting the mean values of tetracycline excreted in one, two, three, and four hours from 200-mg. doses gave the following equation which corresponded to Eq. 10

$$Ae = -2.65t + 6.26t^3 - 1.41t^5 + 0.104t^4 \quad \text{(Eq. 11)}$$

The derivative of Eq. 11 has the value

$$dAe/dt = -2.65 + 12.5t - 4.23t^3 + 0.416t^4 \quad \text{(Eq. 12)}$$

The second derivative has the value

$$d^3Ae/dt^3 = 12.5 - 8.46t + 1.25t^3 \quad \text{(Eq. 13)}$$

The value of Eq. 13 at one and two hours after multiplying by the reciprocal of K, 11.5 (7) is 60.8 and 6.70, respectively. The value of Eq. 12 at the same times is 6.04 and 8.78, respectively. Adding the one hour values just calculated in accordance with Eq. 9, absorption rate expressed as a product with f was about 70 mg. f/hour. At two hours the value was about 15 mg. f/hour, indicating absorption had almost ceased. With tetracycline, f may be estimated to be between 0.5 and 0.7 meaning absorption rate at one hour was between

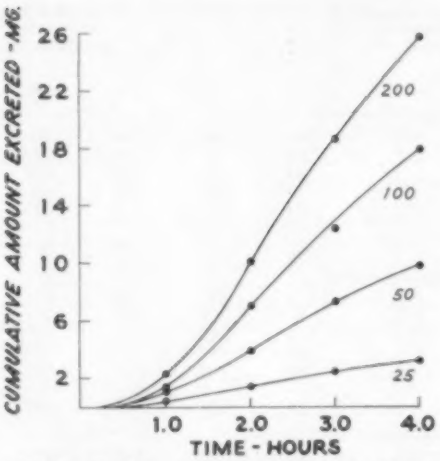

Fig. 1.—Variation in cumulative amounts of tetracycline excreted with time from several doses of tetracycline hydrochloride. Each curve labeled with the amount of tetracycline hydrochloride taken in mg.

TABLE II.—COEFFICIENTS OF THE POLYNOMIAL EQUATION (EQ. 10) FOR VARIOUS DOSES OF TETRACYCLINE HCl[a]

Dose, mg.	a	b	c	d
25	0.100	0.308	0	0
50	−0.252	1.41	−0.151	0
100	−4.10	7.46	−2.31	0.246
200	−2.65	6.26	−1.41	0.104

[a] Entries of zero means less than 0.01.

TABLE III.—COEFFICIENTS OF THE POLYNOMIAL EQUATION (EQ. 10) FOR EACH TEST SUBJECT TAKING 200-MG. DOSES OF TETRACYCLINE HCl

Subject	a	b	c	d
E	−0.942	4.48	−0.759	0.021
S	−0.249	2.35	0.436	−0.137
N	−7.23	15.43	−4.89	0.492
C	−5.31	7.81	−2.14	0.237
J	−0.091	1.91	0.044	−0.063

100 to 140 mg./hour and at two hours between 20 to 30 mg./hour, when 200-mg. doses were taken.

Summary of Data From Various Doses.—The calculated absorption rate as a product with f after the various doses is summarized in Table IV. The same quantity for each test subject after 200-mg. doses were ingested by them is listed in Table V.

TABLE IV.—VALUES OF ABSORPTION RATE AT ONE AND TWO HOURS[a,b]

Time, hr.	Dose 25	50	100	200
1	10	25	52	70
2	10	15	0	15

[a] Expressed as a product with f. [b] To the nearest 5 mg. f/hr.

TABLE V.—VALUES OF ABSORPTION RATE FOR EACH TEST SUBJECT FOLLOWING 200-MG. DOSES OF TETRACYCLINE HCl[a,b]

Time, hr.	G	S	N	C	J	Mean
1	60	70	100	70	40	70
2	20	50	0	20	20	20

[a] Expressed as a product with f. [b] To the nearest 5 mg. f/hr.

It is apparent that wide variation existed in the absorbability of tetracycline hydrochloride by the test subject, but this variation is not unusual in biological experiments. Comparison of the absorption rate values from pooled data following 200-mg. doses (Table IV) to the means of the individually calculated absorption rates (Table V) following the same dose of drug tends to indicate that the averaging of data did not cause excessive error in subsequent mathematical analysis.

No practical alternative procedure to the present method exists to study the absorption mechanism of tetracycline in humans in the range of doses given here. The low tetracycline serum levels resulting from the smaller doses precluded application of the method of Dominguez (1). Analysis for tetracycline in the urine was possible from the smaller doses because the volume of fluid in which excreted material was contained was much smaller than the volume of fluid in which the drug was distributed in the body.

Calculations with Other Drugs.—A comparison of absorption rates of sulfaethylthiadiazole calculated by the method described here and by the Dominguez method (1) has been made (8).

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The Relationship of Metal Binding to the Biological Activities of Phenethylbiguanide*

By EUGENE D. WEINBERG, ROBERT CHERNIN, and JOHN H. BILLMAN

Phenethylbiguanide has been found to form metal complexes with divalent copper and nickel; on the basis of infrared absorption spectra and on theoretical considerations, a revised structure for the metal complexes of biguanides is proposed. Antimicrobial activities of phenethylbiguanide are not affected by divalent copper and nickel but are significantly depressed by iron. The relationship of these findings to the biological mechanisms of action of the compound is discussed.

SUCH POWERFUL metal binding agents as 8-hydroxyquinoline, hematoxylin, and certain thiosemicarbazones are diabetogenic (1, 2). Chemical modification of 8-hydroxyquinoline to prevent metal binding is accompanied by a loss of the ability to cause diabetes (3, 4). It has been suggested that the metal binding compounds act by altering the availability of zinc to the islet cells although the necessity of zinc for either the formation, storage, or functioning of insulin has by no means been established (1, 5, 6).

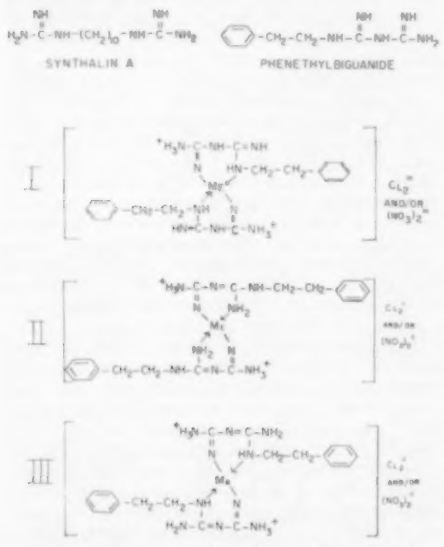

Fig. 1.—Structural formulas of Synthalin A, phenethylbiguanide, and theoretical structural formulas of the metal chelates of phenethylbiguanide.

Certain other strong metal binding agents as isoniazid and the tetracyclines are not diabetogenic, at least at the chemotherapeutic levels ordinarily attained; nor do they appear to affect carbohydrate metabolism in any way (7, 8). Still other metal binding agents, such as the salicylates, are not only nondiabetogenic but can actually reduce the fasting blood sugar and glycosuria of moderately severe diabetic patients (9). A group of metal binding agents that have long been known to possess hypoglycemic activity for diabetic patients are certain diguanidines of which Synthalin A is the outstanding example (Fig. 1). An additional group of hypoglycemic compounds with potential metal binding ability includes 1-(*p*-chlorophenyl)-4-isopropyl biguanide (Paludrine), amylbiguanide, and *β*-phenethylbiguanide (PEBG, DBI). Of the biguanides, PEBG (Fig. 1) has been the most intensively tested for possible use as an oral antidiabetic drug.

The ability of PEBG to form specific metal chelates has not been reported. This paper describes studies of (*a*) the metal binding activity of PEBG and (*b*) the effects of metallic ions on certain antimicrobial properties of this compound

CHEMICAL STUDIES

Aqueous 0.05 *M* solutions of PEBG·HCl[1] were combined with ammoniacal 0.05 *M* to 1.0 *M* solutions of the nitrates of lithium, beryllium, sodium, magnesium, aluminum, potassium, calcium, chromium, manganese, ferrous and ferric iron, cobalt, nickel, divalent copper, zinc, strontium, silver, cadmium, tin, cesium, barium, monovalent and divalent mercury, and lead. In each case, the pH reaction was adjusted to 6–7 with 1.0 *M* NH_4OH, and a blank without PEBG was included to eliminate the possibility that any precipitates that might form might be metal hydroxides rather than metal chelates.

Precipitates of PEBG·HCl plus the inorganic nitrates were obtained only with the salts of copper and nickel. Crystals of the copper and nickel complexes of PEBG were subsequently prepared by digesting the freshly prepared metal hydroxides with PEBG·HCl in a water bath, followed by concentrating and cooling the filtrate. The crystals of the copper and nickel complexes were blue-violet

* Received September 3, 1959, from the Departments of Bacteriology and Chemistry, Indiana University, Bloomington.
Technical assistance of Misses Judith Brooks, Janet Bowers, and Sharron Nance is acknowledged.
This project was supported in part by Grant E-2252 from the U. S. Public Health Service.

[1] Phenethylbiguanide was kindly supplied by the U. S. Vitamin Corp. through the courtesy of Dr. H. S. Sadow.

and pink, respectively. The infrared absorption spectra of a KBr mull of PEBG and the copper and nickel complexes were obtained with an Infracord spectrophotometer with a slit width of 25 μ (Fig. 2).

Biguanide sulfate has been utilized for the determination of divalent copper and nickel with which it forms sparingly-soluble inner metallic complexes of the second order. Such ions as those of zinc, cadmium, magnesium, and the alkali metals do not interfere with this reaction (10). The copper and nickel phenylbiguanides have been described, as have also the metaphenylene, ethylene, α-naphthyl, and β-phenethylbiguanide chelates of these metals (11, 12). The α-copper complex of phenylbiguanide has been reported to be bluish-violet and the β-copper complex to be brick red. The α-nickel complex is brick red and the β-nickel complex light yellow. By color analogy, the PEBG chelates prepared in the present study are probably the α-copper form and the β-nickel form.

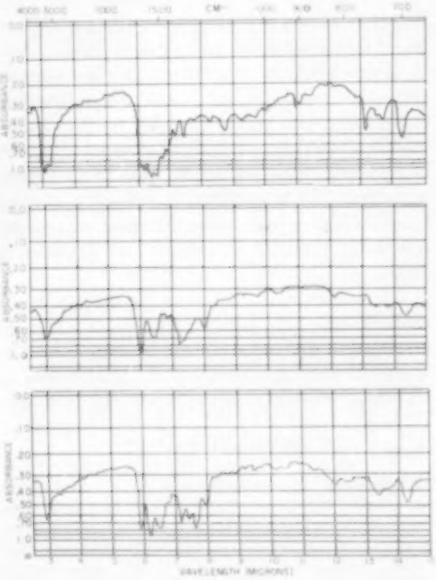

Fig. 2.—Infrared absorption spectra of phenethylbiguanide (top), copper chelate (middle), and nickel chelate (bottom).

According to the studies reported with phenylbiguanide (11), the structures of the PEBG chelates would be represented by I in Fig. 1. However, two additional possible structures are represented by II and III.

It may be noted that the chelate rings in structure I contain only one double bond and that the second double bond is exocyclic. In contrast, the double bonds in structures II and III are both endocyclic. Inasmuch as six-membered chelate rings containing two double bonds are more stable than rings with one endocyclic and one exocyclic double bond, it is considered that structures II or III are more probable than I. The phenethyl group should make the nitrogen in the adjacent amino group more basic than the nitrogen in the imino group and it would therefore be more likely that the former would participate in chelation than the latter. Accordingly, it is suggested that structure III would be more probable than structure II. Unfortunately, the infrared absorption spectra do not indicate which of the chelate structures has actually formed.

MICROBIOLOGICAL STUDIES

Strains of *Serratia*, *Escherichia*, *Bacillus*, *Staphylococcus*, *Saccharomyces*, and *Chlorella* were grown in both complex and minimal synthetic agar media and the microstatic concentrations of PEBG were determined for each strain in each environment. Filter paper disks containing 1×10^{-3} *M* solutions of the respective nitrates listed in the section on chemical studies were placed on inoculated complex and synthetic agar media containing submicrostatic, microstatic, and excess microstatic concentrations of PEBG. In subsequent experiments the copper and nickel complexes of PEBG were tested for microstatic activity.

The minimal microstatic concentrations of PEBG in complex media and the effects of the nitrates of iron on these concentrations are given in Table I. It may be observed that with each microorganism, the activity of PEBG was depressed by iron. No significant depressing or enhancing effects on PEBG were observed with any of the other metallic nitrates tested except that of a slight depressing effect by aluminum with *Escherichia*. Similar minimal microstatic concentrations and depressing effects of iron were observed when the microorganisms were grown in synthetic media. The copper and nickel chelates possessed approximately the same microstatic activity as PEBG in complex media but were five to ten times more active in synthetic media. Presumably the chelates are dissociated by the microorganisms since the apparently enhanced activity in synthetic media was found to be caused by copper or nickel when these metals were tested (as the nitrates) in the absence of PEBG. These metallic ions are not toxic in complex media because of the numerous protective metal binding agents (amino acids, vitamins, etc.) in such media.

Molar concentrations of PEBG between 1×10^{-7} and 1×10^{-5} were tested for microstatic activity to determine the possible existence with this compound of the phenomenon of "concentration quenching." Such metal binding agents as 8-hydroxyquinoline and dimethyldithiocarbamic acid, for example, are more active at lower than at higher concentrations in media containing a constant low amount of iron or copper, respectively (13). In contrast, such metal binding agents as the tetracyclines and bacitracin do not become less active as their concentration is increased in an environment with a fixed concentration of metal ions (14). Like the antibiotics, PEBG did not exhibit "concentration quenching"; the drug was completely inactive at levels below 1×10^{-3} *M* with each test genus. Despite the fact that these results indicate that PEBG does not appear to require an extracellular equimolar concentration of a metallic ion for microstatic activity, it is quite possible that the molecules of the drug that enter the cell can form che-

TABLE I.—EFFECT OF METALLIC IONS ON THE MINIMAL MICROSTATIC CONCENTRATIONS OF PHENETHYLBIGUANIDE

Genus	Disk	Concentration of PEBG, × 10^{-3} M[a]							
		0	1	2	3	4	5	10	20
Serratia	Deionized H_2O	C	C	..	..	..	..	..	..
	Fe^{++} or Fe^{+++}	C	C	+	+	+	..	..	..
	Other 22 cations	C	C	..	..	..	..	..	..
Escherichia	Deionized H_2O	C	C	..	..	..	..	..	..
	Fe^{++} or Fe^{+++}	C	C	+	+	..	..	..	..
	Other 22 cations[a]	C	C	..	..	..	..	..	..
Bacillus	Deionized H_2O	C	C	C	C	..	..	..	..
	Fe^{++} or Fe^{+++}	C	C	C	C	+	..	..	..
	Other 22 cations	C	C	C	C	..	..	..	..
Staphylococcus	Deionized H_2O	C	C	..	..	..	..	..	..
	Fe^{++} or Fe^{+++}	C	C	+	..	..	..	..	..
	Other 22 cations	C	C	..	..	..	..	..	..
Saccharomyces	Deionized H_2O	C	C	..	..	..	..	..	..
	Fe^{++} or Fe^{+++}	C	C	+	+	+	..	..	..
	Other 22 cations	C	C	..	..	..	..	..	..
Chlorella	Deionized H_2O	C	C	C	C	C	..	..	..
	Fe^{++} or Fe^{+++}	C	C	C	C	C	+	+	+
	Other 22 cations	C	C	C	C	C	..	..	..

[a] Complete growth throughout plate: C, many colonies around disk (no growth in remainder of plate); +, no colonies around disk or in remainder of plate except for aluminum which allowed colonies to appear at a concentration of 2 × 10^{-3} M.

lates with intracellular metal ions and that a 1:1 complex might be the actual toxic entity for specific enzymatic systems.

In other experiments, the effect of PEBG on the requirement of zinc for (*a*) the antibacterial action of bacitracin and (*b*) the production of prodigiosin by certain strains of *Serratia* was studied and compared with the effect of ethylenediaminetetraacetic acid (EDTA) on these zinc-dependent functions. In contrast to EDTA which can depress the action of bacitracin by depriving the antibiotic of zinc (14), PEBG had no effect on the antibacterial action of the drug (Fig. 3). Both EDTA and PEBG prevented the formation of prodigiosin by zinc-dependent strains of *Serratia*, but excess zinc was not

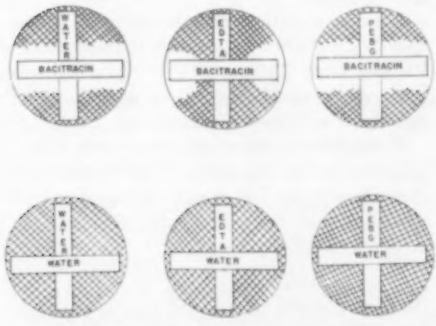

Fig. 3.—Extent of visible growth in nutrient agar plates seeded with *Staphylococcus aureus* after two days at 37°. The shaded areas represent areas containing bacterial colonies. The rectangles represent filter paper strips that were briefly soaked in the material stated on the specific rectangle and then placed on the inoculated agar at the beginning of the period of incubation. The concentration of each of the solutions of bacitracin, PEBG, and EDTA was 1 × 10^{-3} M.

able to suppress this action of either of the metal binding agents. In contrast, ferrous iron (which, like zinc, is required for prodigiosin production) was able to reverse the action of both EDTA and PEBG.

Although previous and present chemical studies with biguanides indicate that stable chelates are formed mainly with copper and nickel, the previous and present biological studies indicate that iron-dependent enzymatic systems may be affected by PEBG. The compound has been reported to inhibit a cytochrome oxidase system of rat liver homogenates (15) and to suppress a portion of the electron transport system before cytochrome b of rat liver mitochondria (16). Many portions of this part of the electron transport system are activated by iron (17). This situation may be analogous to that of the tetracyclines which form strong chelates with copper and nickel but which attack iron-dependent enzymes; as with PEBG, iron rather than copper and nickel strongly affects the actions of the tetracyclines (18).

It is apparent from the previous and present biological studies with PEBG that zinc is not involved in the activities of the compound. And since the hypoglycemic action of PEBG is apparently not associated with the utilization of insulin (16), such a result is not unexpected.

In subsequent studies with PEBG it is obvious that the chelating ability of the compound should be considered as both a possible beneficial and a detrimental characteristic of the drug. For example, might side effects of the compound be eliminated by including an iron salt in the PEBG formulation? Or, would a strong chelating agent such as a salt of EDTA potentiate the action of PEBG by reducing the amount of iron available to neutralize the drug? Many additional questions of this nature can be raised, and investigations designed to answer these questions will undoubtedly aid in the more efficient chemotherapeutic usage of the drug as well as in the ultimate elucidation of its biochemical mechanism of action.

SUMMARY

1. Evidence is presented for the formation of metal complexes of phenethylbiguanide with copper and nickel.

2. Antimicrobial activities of phenethylbiguanide are not affected by copper or nickel but are depressed by iron.

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The Solubility of Benzoic Acid in Mixed Solvents*

By MARVIN J. CHERTKOFF† and ALFRED N. MARTIN

The solubility of benzoic acid was determined in mixtures of several solvents of varying polarity. The experimental results were compared with the theoretical solubility as calculated from the Hildebrand equation for regular solutions. The theoretical and experimental results agree well in the region where the solubility parameter of the solute approaches that of the solvent mixture.

MOST LIQUID PHARMACEUTICALS consist of one or more solids dissolved in a mixture of liquids. The solubility of the solids varies with the composition of the solvent mixture, therefore, information which would allow the pharmacist to control the solvent properties of a mixture of liquids would be of great value.

This investigation was undertaken to study the solubility of a solute in a series of mixed solvent systems. Benzoic acid was chosen as a prototype of relatively polar pharmaceutical solids and the solvents were selected to provide a continuous spectra of increasing polarity from hexane to water. The experimental findings were analyzed based on the polarity of the solvent mixture, utilizing the Hildebrand equations for solubility.

A solution which obeys Raoult's law is known as an ideal solution. The solubility of the solute in such a system may be calculated from the heat of fusion of the solute and the heat capacities of this solid and of its supercooled liquid. Hildebrand and Scott (1) derived such a relationship,

$$\log x_2 = -\frac{\Delta H_m^F}{4.575}\left(\frac{T_m - T}{TT_m}\right) + \frac{\Delta C_p}{4.575}\left(\frac{T_m - T}{T}\right) - \frac{\Delta C_p}{1.987}\log\left(\frac{T_m}{T}\right) \quad \text{(Eq. 1)}$$

where x_2 is the mole fraction solubility at temperature T, ΔH_m^F the heat of fusion of the solute at its melting point T_m, and $\Delta C_p = C_p^l - C_p^s$ where C_p^l and C_p^s are the molal heat capacities of the liquid and solid forms, respectively.

For regular solutions (2), those in which heat is absorbed on mixing but no entropy change occurs, short range interactions between the components must be considered. Equation 1 is modified (3) to Eq. 2

$$\log x_2 = -\frac{\Delta H_m^F}{4.575}\left(\frac{T_m - T}{T_m T}\right) + \frac{\Delta C_p}{4.575}\left(\frac{T_m - T}{T}\right) - \frac{\Delta C_p}{1.987}\log\frac{T_m}{T} - \frac{V_2}{4.575\,T}(\delta_1 - \delta_2)^2\,\phi_1^2 \quad \text{(Eq. 2)}$$

in which V_2 is the molar volume of the solute, ϕ_1 is the volume fraction of the solvent, and δ_1 and δ_2 are the "solubility parameters" of the solvent and solute, respectively. The solubility

* Received August 21, 1959, from the School of Pharmacy, Purdue University, Lafayette, Ind.
Supported in part by a grant from the Purdue Research Foundation.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
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parameter, as defined by Hildebrand and Scott (4), is the square root of the internal pressure of the substance, a measure of its polarity, so that the more polar a substance the greater its δ value. Although Hildebrand restricts the use of his equation to nonpolar solvents, an attempt is made in a later part of this communication to show that the equation for regular solutions is applicable in some systems when the polarity of the solvents are not significantly different from one another.

For two component solvent systems, ϕ_1 is taken as the volume fraction of the combined solvents a and b, and the new solubility parameters for the mixed solvents may be calculated from

$$\delta_1 = \frac{\phi_a \delta_a + \phi_b \delta_b}{\phi_a + \phi_b} \qquad \text{(Eq. 3)}$$

where $\phi_1 = \phi_a + \phi_b$.

EXPERIMENTAL

Reagents.—Benzoic acid, Fisher certified reagent A. C. S. grade (A-65), recrystallized from chloroform; ethyl acetate, Fisher certified reagent A. C. S. grade (E-145); carbon tetrachloride, spectroanalyzed, Fisher certified reagent A. C. S. grade (C-199); hexane, Viking petroleum solvents, United Fuel Gas Co., Charleston, W. Va., boiling point 65–67°; distilled water, deionized by passage through IRA 120 and IRA 400 resins; commercial absolute ethanol, redistilled and dried over 20-mesh Drierite (5).

Procedure.—A slight excess of solid was added to a given volume of the solvent mixture contained in a screw-top bottle. A closure was made with aluminum foil, the top fixed tightly over the foil, and the whole sealed with several turns of electrical tape. The bottles were shaken in a constant temperature bath (Waco Aquarium Vis bath) at 25 ± 0.1° for twenty-four hours. The equilibrated solutions were then removed, filtered, and samples taken.

The saturated solutions were suitably diluted with 95% ethanol, and the concentration of the benzoic acid was determined spectrophotometrically on a Beckman model DU spectrophotometer at 272.5 mμ.

Densities of the saturated solutions were determined gravimetrically.

CALCULATIONS

The ideal solubility of benzoic acid was calculated from Eq. 1 using the following data: $\Delta H_m^F = 4302$ cal./mole (6), $T_m = 395.6°$ (7), and $\Delta C_p = C_p^l - C_p^s$ (at the melting point) = 13.83 cal./degree/mole (7).

For the calculated solubilities in different solvent mixtures, Eq. 2 was used. The density of the supercooled liquid benzoic acid at 25° was not available in the literature, however the density of the liquid had been reported at several temperatures above its melting point. A plot of density against temperature

Temperature, °C.	Density, Gm./cc.	Reference
122.5	1.080	(8)
125.0	1.077	(9)
130.0	1.078	(8)
130.0	1.075	(10)
131.9	1.074	(11)
155.0	1.052	(10)
180.0	1.029	(10)

gave a linear relationship, and by the method of least squares, the equation for the regression line was found to be

$$d = 1.1926 - (9.064 \times 10^{-1})\, t$$

The density at 25° was calculated to be 1.1699 Gm./cc., which gave a molar volume of 104.38 cc./mole.

The solubility parameters used were as follows: Hildebrand and Scott (12) gave $\delta = 7.3$ for hexane and $\delta = 8.6$ for carbon tetrachloride. The solubility parameters for *n*-propyl, amyl, *n*-hexyl, *n*-heptyl, and *n*-octyl alcohol were taken from Hildebrand and Scott (13), and those for methyl and *n*-butyl alcohol were calculated from their internal pressures (14). These values were plotted against number of carbon atoms and a smooth curve was fitted. This line crossed the two carbon coordinates at a solubility parameter value of 13.0 which was used for ethyl alcohol.

The solubility parameter of benzoic acid was calculated as 11.5 from the solubility data in hexane. The values for water and ethyl acetate were calculated from the equation (15)

$$\delta = \left(\frac{\Delta H^v - RT}{V^l}\right)^{1/2} \qquad \text{(Eq. 4)}$$

and were found to be 23.4 and 9.0, respectively.

In Eq. 4, ΔH^v is the molar heat of vaporization, V^l is the molar volume at 25°, R is the gas constant, and T is the absolute temperature. For water, the heat of vaporization at 25° was taken as 582.2 cal./Gm., an average of the values at 20° and 30° (16).

For ethyl acetate, the following heats of vaporization were plotted and the value at 25° read as 97.5 cal./Gm.:

Temperature, °C.	ΔH^v, cal./Gm.	Reference
0.0	102.00	(17)
76.0	87.63	(18)
76.5	87.97	(19)

An example of these calculations for the solubility of benzoic acid in a mixture of equal volumes of ethyl acetate and hexane at 25° follows:

From Eq. 3

$$\delta_1 = \frac{\phi_a \delta_a + \phi_b \delta_b}{\phi_a + \phi_b} = \frac{(0.5 \times 7.3) + (0.5 \times 9.0)}{0.5 + 0.5} = 8.15$$

Then employing Eq. 2

$$\log x_2 = -\frac{4302\,(395.6 - 298.2)}{4.575 \times 395.6 \times 298.2} + \frac{13.83\,(395.6 - 298.2)}{4.575 \times 298.2} - \frac{13.83}{1.987} \log \frac{395.6}{292.2} - \frac{104.38\,(11.5 - 8.15)^2\,(1)^2}{4.575 \times 298.2}$$

$$\log x_2 = 1.5014 \quad x_2 = 0.03152$$

In the above calculation, ϕ_1 was taken as equal to one. In more concentrated solutions this introduces a substantial error, so that the method of successive approximations was applied to obtain a theoretical ϕ_1. Using Eq. 5, which was obtained by combining

$$\phi_1 = \frac{(1 - x_2)\, V_1}{(1 - x_2)\, V_1 + x_2 V_2} \qquad \text{(Eq. 5)}$$

the equations for volume fraction and mole fraction, and the value for x_2 obtained above, a new ϕ_1 was calculated which was then used in Eq. 2 to recalculate x_2. This procedure was repeated several times until x_2 did not change significantly and a final theoretical mole fraction solubility was obtained. For practical work these values would, of course, be converted to the molal scale, or knowing the density of the solution, to molarity.

The values for V_1, the molar volume of the solvent, were calculated as weighted averages of the molar volume of the components of the solvent mixture in a manner similar to that used for the calculation of δ values of the mixed solvents. The error introduced by the assumption that molar volumes are additive was accepted to simplify the calculations.

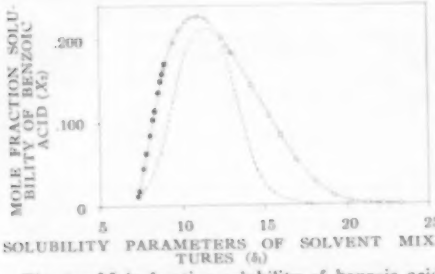

Fig. 1.—Mole fraction solubility af benzoic acid *vs.* solubility parameters of the solvent mixtures hexane–ethyl acetate, ethyl acetate–ethyl alcohol, ethyl alcohol–water. —— observed, ● hexane–ethyl acetate, □ ethyl acetate–ethyl alcohol, ○ ethyl alcohol–water; - - - calculated.

DISCUSSION

From Eq. 2 it is obvious that when $\delta_1 = \delta_2$, the last term becomes equal to zero, Eq. 2 becomes identical with Eq. 1, and the ideal solubility of the solute should be observed. If a pair of miscible liquids are chosen so that their solubility parameters lie on either side of that for a solute, the volume fractions of the two which will make $\delta_1 = \delta_2$ can be calculated from Eq. 3. Such a mixture would be an "ideal" solvent for that solute; that is, the solute would exhibit the solubility predicted by the ideal solubility equation, provided the solution meets the criteria of a regular solution as defined by Hildebrand and Scott. Also, since the last term of Eq. 2 is negative, its presence will lower the value of x_2; when this last term is equal to zero, the maximum solubility should be observed.

Figure 1 shows the results of the calculated and observed mole fraction solubilities of benzoic acid (x_2) *versus* the solubility parameters of the solvent mixtures ethyl acetate–hexane, ethyl acetate–ethanol, and ethanol–water. The calculated curve reached a maximum peak at $\delta = 11.5$, since this was the solubility parameter for benzoic acid. The experimental curve exhibited a plateau region from about 10.2 to 11.8.

The similarity of these two curves is surprising, since such excellent agreement is not often observed in solubility work; furthermore, the high polarity and hydrogen bonding in the system makes these results all the more interesting. The calculated ideal solubility of benzoic acid at $\delta_1 = 11.5$ ($x_2 = 0.2275$) lies on the experimental curve. Gordon and Scott (20) investigated a nonpolar system, phenanthrene dissolved in a mixture of methylene iodide and cyclohexane having various δ values, and found that while the maximum solubility occurred where $\delta_{solvent} \cong \delta_{solute}$, the experimental solubility was considerably below that of the ideal value.

In the present study, good agreement was found between observed and calculated solubility from about 50 to 100% v/v ethyl alcohol in ethyl acetate (δ = 11.0 to 13.0). As the concentration of ethyl acetate increased, the theoretical and experimental values no longer coincided, as observed in the left hand portion of the curve in Fig. 1. The same behavior was observed as water was added, that is, for δ values of 14.0 to 23.4. Interactions between the solute and these solvents would cause a change in entropy, creating a nonregular solution, and thus the Hildebrand treatment would not be expected to apply. In view of this, the surprising fact is not that deviation occurred over most of the curves, but that the curves coincided in the region of the maximum (δ = 11.5). Perhaps the magnitude of the interactions in the peak area were such that deviations from regular solution theory nullified one another.

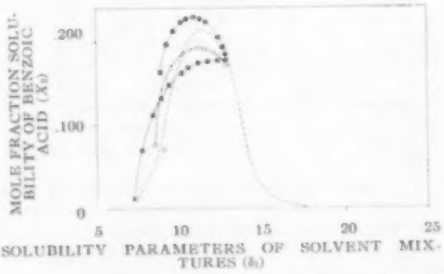

Fig. 2.—Mole fraction solubility of benzoic acid *vs.* solubility parameters of the solvent mixtures hexane–ethyl alcohol, carbon tetrachloride–ethyl alcohol, ethyl acetate–ethyl alcohol. —— observed, ■ hexane–ethyl alcohol, ○ carbon tetrachloride–ethyl alcohol, ● ethyl acetate–ethyl alcohol; - - - calculated.

Additional experimentation was conducted to verify these findings. Systems were chosen so that the δ values of the two liquids in a given solvent mixture bracketed the δ value of benzoic acid. Figure 2 demonstrates the solubility obtained for benzoic acid in three mixed solvent systems. In each case, ethanol was taken as the solvent with the δ value (13.0)

higher than that of benzoic acid (11.5) since it was completely miscible with all the other liquids used in this study. As the solubility parameters of the low polarity solvents, hexane (7.3), carbon tetrachloride (8.6), and ethyl acetate (9.0) approached that of ethanol (13.0), the curve of experimentally determined solubility approached the theoretical curve. In the hexane–ethanol system, neither qualitative nor quantitative agreement of experimental and theoretical results was observed. Qualitative agreement was found in the carbon tetrachloride–ethanol system, that is, the maximum solubility was observed at approximately the theoretically predicted value, however, quantitative agreement was not good.

In the ethyl acetate–ethanol system both qualitative and quantitative correlations were obtained. From a practical standpoint, these results suggested that in order to attain the maximum solubility of a compound in a mixed solvent system, the solvents should not only provide a δ value equal to that of the solute, but furthermore, the individual solvents of the mixture should not be too widely separated in polarity. Further investigations on the application of the Hildebrand formulation to solutions of moderately high polarity must be conducted to determine if the correspondence observed in these systems occurs with other polar solvents and solutes.

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The Photo-oxidation of Dihydroxyphenylalanine in the Presence of 8-Methoxypsoralen*

By JOSEPH JUDIS

8-Methoxypsoralen (8-MOP) is well known for its ability to stimulate melanin formation in vitiligo or normal skin if administration is coupled with exposure to sunlight or ultraviolet light. As an approach to studying the mechanism of action of 8-MOP, the latter was incubated with DOPA and exposed to various types of light. Sunlight, white light, or long wave ultraviolet light (3660 Å.) stimulated the oxidation of DOPA in the presence of 8-MOP but not in the absence of the latter. 8-MOP protected DOPA against short wavelength ultraviolet (2540 Å.) although DOPA was oxidized by the latter in the absence of 8-MOP. The photo-oxidation of DOPA in the presence of long wavelength ultraviolet light and 8-MOP was more rapid at pH values above 7 and was completely inhibited by ascorbic acid, cysteine, cysteamine, β-aminoethylisothiuronium bromide HBr, and partially inhibited by sodium thioglycollate. The possible implication of these *in vitro* observations to the mode of action of 8-MOP *in vivo* is discussed.

A CRYSTALLINE PRINCIPLE, 8-methoxypsoralen (8-MOP),[1] found in the fruit of the plant *Ammi majus* Linn., is one of the several furocoumarins recently isolated from this source (1). At the present time, 8-MOP is widely used, both orally and topically, as a treatment for vitiligo (2, 3) as well as for the promotion of suntanning in normal persons (4, 5, 6).

While it has been clearly established that 8-MOP stimulates pigmentation of the skin, its mechanism of action is not understood. Pigmentation is due to the formation of melanin by a number of steps (7–10) beginning with tyrosine. The classic scheme (10) for the enzymatic oxidation of tyrosine consists of the following steps: (*a*) oxidation in the presence of tyrosinase to

* Received August 21, 1959, from the College of Pharmacy, University of Toledo, Toledo, Ohio.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

[1] Hereafter, 8-methoxypsoralen will be abbreviated as 8-MOP, and dihydroxyphenylalanine will be abbreviated as DOPA.

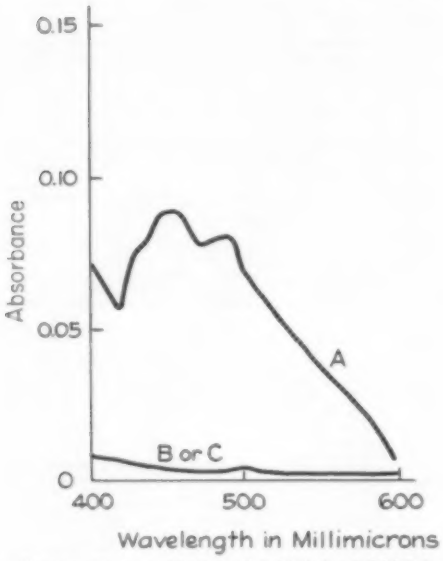

Fig. 1.—Photo-oxidation of DOPA by 8-MOP in white light. *A*, DOPA plus 8-MOP in white light; *B*, DOPA without 8-MOP in white light; *C*, DOPA plus 8-MOP in the dark. Time of irradiation: forty-five minutes.

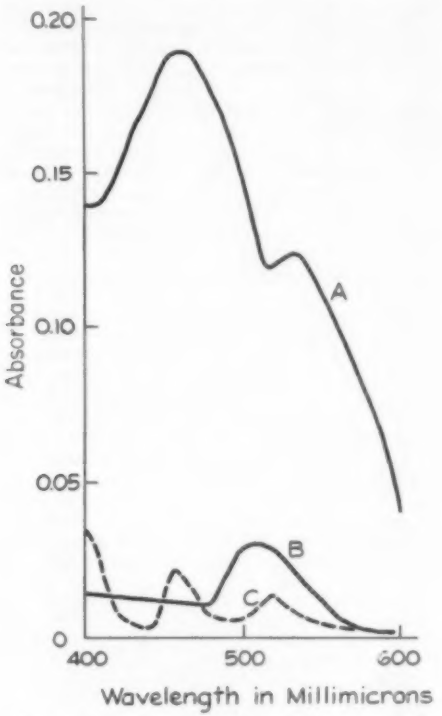

Fig. 2.—Photo-oxidation of DOPA by 8-MOP in "black light." *A*, DOPA plus 8-MOP in "black light;" *B*, DOPA without 8-MOP in "black light;" *C*, DOPA plus 8-MOP in the dark. Time of irradiation: Thirty-five minutes.

DOPA; (*b*) further oxidation of DOPA by tyrosinase to DOPA quinone; (*c*) condensation to a leuco compound; (*d*) oxidation to DOPA chrome; (*e*) decarboxylation and reduction to 5,6-dihydroxyindole; (*f*) oxidation to indole 5,6-quinone; (*g*) polymerization and oxidation to DOPA melanin. *In vivo*, the quinones would react readily, before or after polymerization, with the SH and NH_2 groups of proteins to form a melanoprotein compound.

It is well known that the sequence of events leading to melanin formation is influenced by a number of factors such as copper, an essential part of tyrosinase; sulfhydryl groups, which inhibit and thus regulate the tyrosine-tyrosinase reaction; various hormones such as the melanocyte-stimulating hormone (MSH), thyroid, adrenal, and gonadal; temperature; electrolyte concentration; pH and oxidation-reduction potential. Since the stimulatory effect of 8-MOP on melanin formation is clearly a photochemical one, it seemed of interest to see whether a key compound, such as tyrosine or DOPA could be photochemically oxidized to melanin, using 8-MOP as the photosensitizer. In preliminary experiments, tyrosine was not photo-oxidized under the conditions used but DOPA was, so the photo-oxidation of the latter compound was studied.

MATERIALS AND METHODS

Materials.—DOPA was obtained from the California Corp. for Biochemical Research or Nutritional Biochemicals Corp. The ascorbic acid, cysteine hydrochloride, and cysteamine hydrochloride were obtained from the California Corp. for Biochemical Research. The disodium versenate was supplied by Dow Chemical Corp., the sodium thioglycollate was obtained from Baltimore Biological Laboratories, and the AET (β-aminoethylisothiuronium bromide hydrobromide) was purchased from Schwartz Laboratories. The 8-MOP was used as the 1% lotion in isopropanol, acetone, and alcohol marketed by the Paul B. Elder Co., Bryan, Ohio, under the trademark of Oxsoralen. The other psoralen derivatives were a gift of the Paul B. Elder Co. The phosphate buffers of various pH's were prepared by mixing the proper proportions of 0.067 *M* KH_2PO_4 and 0.067 *M* Na_2HPO_4.

The artificial light sources were as follows: (*a*) white light, two 150-watt Sylvania flood lamps; (*b*) long wavelength ultraviolet, G. E. 15-watt "black light" emitting most of its radiations at approximately 3660 Å.; (*c*) short wavelength ultraviolet, G. E. 15-watt germicidal lamp emitting most of its radiation at 2540 Å.

Procedure.—The typical experimental procedure was as follows: the reaction mixtures were placed in 16 × 150 mm. Kimax screw-cap test tubes at a distance of 10 cm. from the light source in the case of irradiation with "black light" or white light. Absorbance readings were made on the Spectronic 20 spectrophotometer. Absorption spectra were usually performed on the Beckman DU quartz spectrophotometer using the appropriate 1-cm. silica cells. When the G. E. germicidal lamp was used instead of the "black light," quartz test tubes, approximately 15 × 150 mm, were substituted for the Kimax test tubes. In irradiating with white light, the test tubes were kept 18 cm. from the light source to allow for a water filter for absorption of the heat produced.

The reaction mixtures normally contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol, and enough 0.067 *M* phosphate buffer of the appropriate pH to make a total of 6.0 ml. If other ingredients were in the reaction mixture, an equivalent volume of the buffer was omitted.

RESULTS AND DISCUSSION

Lerner and his co-workers (11) incubated ultraviolet irradiated 8-MOP with tyrosinase and either tyrosine or DOPA. They found no effects on oxygen uptake or pigment formation. In the present study, DOPA and 8-MOP were incubated together and irradiated. Figure 1 gives the absorption spectrum curve of a reaction mixture irradiated with white light. The amount of pigment formed is not nearly so great as when "black light" is used as the source of light energy (see Fig. 2). With short wavelength ultraviolet light, the absorption curves (Fig. 3) of the reaction mixtures seem to indicate that the 8-MOP protects the DOPA against the ultraviolet light. This is a reasonable explanation since it is known from the ultraviolet absorption curve of 8-MOP (12) that the latter does absorb ultraviolet light at approximately 250 mμ as well as at approximately 300 mμ.

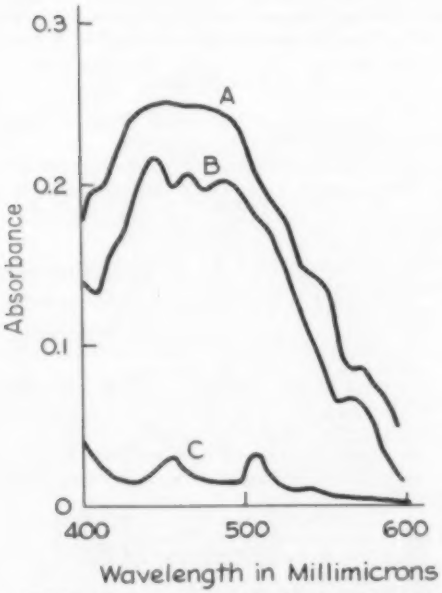

Fig. 3.—Photo-oxidation of DOPA in ultraviolet light. *A*, DOPA without 8-MOP in ultraviolet light; *B*, DOPA plus 8-MOP in ultraviolet light; *C*, DOPA plus 8-MOP in the dark. Time of irradiation: Thirty-five minutes.

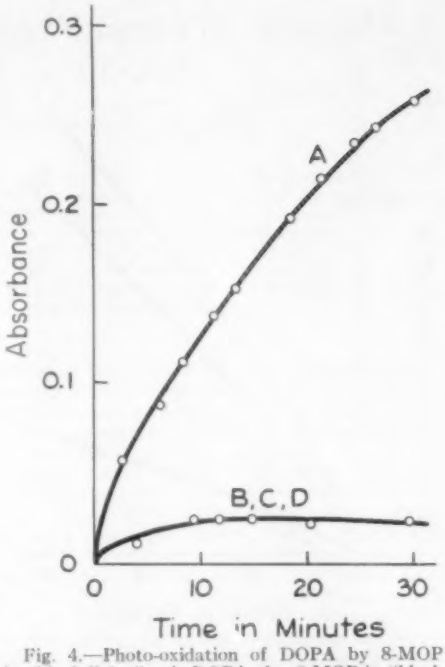

Fig. 4.—Photo-oxidation of DOPA by 8-MOP in "black light." *A*, DOPA plus 8-MOP in "black light;" *B*, DOPA without 8-MOP in "black light;" *C*, DOPA plus 8-MOP in the dark; *D*, 8-MOP in "black light." Absorbance was measured at 480 mμ.

Photo-oxidation of DOPA proceeded rather rapidly with long wavelength ultraviolet as the source of radiant energy. A typical kinetic picture is shown in Fig. 4. A similar kinetic picture was obtained with two other psoralen derivatives, 8-isoamyleneoxypsoralen and 2′,8-dimethylpsoralen.

The effect of pH on the photo-oxidative reaction was found to be similar to that obtained by Hirsch (13) who studied DOPA auto-oxidation as a function of pH. He found melanin yields from DOPA in a given time period to increase as the pH increased. The effect of pH on the photo-oxidation of DOPA is shown in Fig. 5.

There has been a great deal of study on the ability of certain pure chemicals and tissue and blood constituents to inhibit the auto-oxidation of DOPA (14–17). Various workers feel that the body has a chemical mechanism for regulating DOPA oxidation as well as the oxidation of other physiological phenols. This mechanism appears to involve —SH

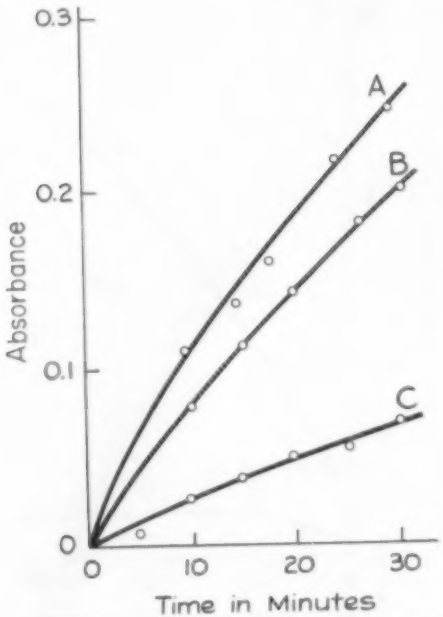

Fig. 5.—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light." *A*, pH 8.05; *B*, pH 6.6; *C*, pH 5.3. Absorbance was measured at 480 mμ.

groups. Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation. Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation-induced DOPA oxidation may operate by one or both of two mechanisms: (*a*) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (*b*) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation. While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals. It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation. A couple of compounds believed to be radioprotective by a chelating mechanism were also included. Table I gives the results obtained.

It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22). Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known. Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8). The mechanism by which 8-MOP may stimulate the suntanning process could possibly be due to its

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit[a]
KCN	0.03 *M*	−
Disodium versenate	0.02 *M*	−
Ascorbic acid	0.002 *M*	+
Cysteine HCl	0.005 *M*	+
Sodium thioglycollate[b]	0.01 *M*	+
Cysteamine HCl	0.01 *M*	+
AET	0.01 *M*	+

[a] The reaction mixtures contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol, an appropriate amount of the test substance dissolved in pH 6.3, 0.067 *M* phosphate buffer, and enough phosphate buffer to make 6.0 ml.
[b] Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome.

accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed. In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome. This is presumably due to the photo-oxidation of sodium thioglycollate.

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24). In this disease, it is possible that 8-MOP may act in several ways: (*a*) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (*b*) to photo-oxidize any quantities of DOPA present to melanin, (*c*) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation. All of these speculations remain to be proved.

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Effects of Fatty Acids on Vitamin A Esters in Isopropanol Solutions*

By ALBERT J. FORLANO† and LOYD E. HARRIS

Vitamin A ester degradation was studied in isopropyl alcohol, isopropyl alcohol and water, cyclohexane, and in these solvents containing members of the acetic acid series of fatty acids. The results indicate that (*a*) the principal methods of degradation are oxidation and elimination in hydroxylated solvents, (*b*) water increases the rates of elimination, (*c*) fatty acids decrease the rates of elimination, and (*d*) oxidation is the main route of decomposition in hydrocarbon solvents. The stabilizing mechanism of the fatty acids was studied by examining the effects of fatty acids, water, and isopropyl alcohol on anhydrovitamin A.

VITAMIN A esters were unstable in vanishing creams containing stearic acid. The nature of this instability has not been established. The study reported herein was undertaken to determine whether fatty acids caused this instability and the nature of the decomposition. Since vitamin A is known to be sensitive to mineral acids (1, 2), it was assumed that stearic acid was the causative agent. That the rate was slower in the presence of fatty acids appeared to be related to the smaller (H^+) liberated by the fatty acids.

Vitamin A palmitate degradation was initially studied in *n*-hexane and isopropanol, both containing 5 per cent stearic acid. In *n*-hexane vitamin A was lost mainly through oxidation; however in isopropanol, both elimination and oxidation occurred. A plot of log concentration *versus* time was a straight line indicative of a first-order dependence on the vitamin A concentration. Some samples were chromatographed on Woelm alumina, deactivated by the addition of 6 per cent water.

EXPERIMENTAL

Materials Used in This Study.—Isopropanol (81–83°) Sohio; Fisher reagent grade stearic acid (68–69.5°); Fisher reagent grade palmitic acid (61–62°); Fisher laboratory chemicals oleic and caproic acids (purified); Eastman Kodak decanoic acid; Dupont reagent glacial acetic acid (99.7%); Mallinckrodt anhydrous ethyl ether; heavy liquid petrolatum U. S. P.; *n*-hexane (technical) OSU label (redistilled); Woelm neutral activated alumina for chromatography (M. Woelm, Eschwage, Germany); vitamin A acetate and palmitate, Hoffmann-La Roche, Inc.; and butylated hydroxy toluene (BHT), food grade, Koppers Co., Inc.

Stability of Vitamin A in the Presence of a Homologous Series of Fatty Acids in Isopropanol.—The purpose of this study was to determine (*a*) if they would accelerate destruction of vitamin A esters, (*b*) if there was any difference in the rates of destruction among the members of a homologous series of fatty acids, (*c*) the nature of decomposition.

Isopropanol was chosen as the solvent because it is water miscible, good solvent for vitamin A esters and the fatty acids, and had only a slight destructive action on vitamin A (3). In order to determine the rates of decomposition and order of dependence on vitamin A, the other ingredients were in a large excess of the vitamin A concentration. Fatty acid concentrations of 0.033 *N* were used because mineral acids in the same concentrations were effective in causing elimination of vitamin A esters and alcohol (2).

Preparation of Solutions.—The distilled isopropanol was flushed with dry nitrogen for one hour. The water content of this solvent was calculated at 0.056% by a Karl Fischer titration (4). Calculated quantities of caproic, decanoic, palmitic, stearic, and oleic acids were added to make 0.033 *N* solutions and enough vitamin A palmitate was added to make an approximate concentration of 10,000 u./ml. A control, not containing fatty acid, was also prepared. The solutions were placed into 120-ml. glass-stoppered amber bottles and their vitamin A potency was determined by the British Pharmacopoeia method (5). The air in these containers was displaced by nitrogen. These containers were subsequently stored at 45, 37, and 25°. When these solutions were titrated with base, it was found that none of the fatty acids were lost through esterification with the isopropanol. The U. V. absorption of the fatty acids did not interfere with the spectrum of the vitamin A. The rates of decomposition are given in Table I. Figure 1 represents the plot of log concentration of vitamin A *versus* time. The control graph is submitted as representative of the entire group.

This graph indicates a pseudo first-order reaction depending only on the concentration of vitamin A ester. The rate constants, except for a few cases (k_1 for stearic, palmitic, and oleic acids at 45° only), indicated that the rate of destruction was slower in the presence of fatty acids and also that the amount of anhydrovitamin A was smaller in the presence of fatty acids. The unexpected drop in the 45° curve (Fig. 1) was due to oxidation which was seen in the entire series. The first three hundred hours could well be considered an induction period before oxidation began. The U. V. extinction at

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Based, in part, upon a Ph.D. dissertation submitted to the Graduate School, Ohio State University.
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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

TABLE I.—RATE CONSTANTS FOR DEGRADATION OF VITAMIN A IN ISOPROPANOL SOLUTION

($k/2.303$ hr.$^{-1} \times 10^4$)

	Isopropanol Control	0.033 *N* Caproic Acid	0.033 *N* Decanoic Acid	0.033 *N* Palmitic Acid	0.033 *N* Oleic Acid	0.033 *N* Stearic Acid
45°						
k_1	13.7	12.0	12.5	14.9	15.0	15.0
k_2	1.50	1.00	1.13	1.05	1.33	1.25
37°						
k_1	12.0	7.70	5.30	5.90	7.01	8.00
k_2	1.25	0.980	0.938	0.833	0.910	0.834

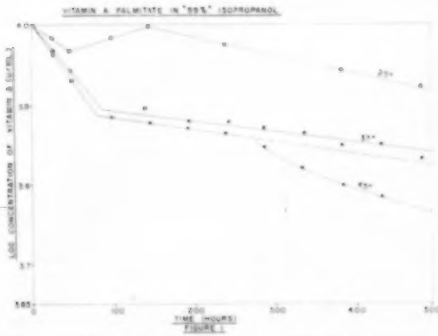


Fig. 1.—Vitamin A palmitate in "99%" isopropanol.

280 mμ rose sharply at the same time the unexpected drop in potency was observed. The hump in the 25° curve is unexplainable even after careful consideration of the data collected.

Chromatography.—The reaction mixtures were chromatographed on Woelm alumina deactivated by the addition of 6% water. Ninety-four grams of activated alumina was placed in a glass-stoppered flask containing 6 Gm. of water and the mixture was allowed to hydrate for two hours, enough purified *n*-hexane was added to the alumina to make a slurry. This slurry was poured into a glass column to a height of 10 inches of alumina.

The isopropanol reaction mixtures were dried *in vacuo* and the residue was dissolved in 5 ml. of purified *n*-hexane. The solution was poured into the column and was immediately followed by *n*-hexane so that the column would not dry out. The weakest eluant, *n*-hexane, was added first and 25-ml. fractions were collected. Anhydrovitamin A, the first substance eluted, was followed by vitamin A esters, both being eluted by *n*-hexane. The following eluate fractions contained very little material as determined by the U. V. absorption; consequently it was necessary to use stronger eluants (2, 4, 8, 16, 25, 40, 50, and 75% ethyl ether in *n*-hexane) to remove the substances remaining on the column.

The U. V. spectra of each of these fractions were determined on a Beckman DK spectrophotometer. Each fraction was subsequently dried *in vacuo* and their I. R. spectra were determined using a Baird infrared spectrophotometer between sodium chloride plates.

The following substances were identified and were eluted in this order: anhydrovitamin A (max. 351 mμ, 371 mμ, and 391 mμ); vitamin A esters (max. 326 mμ); esterified oxidation products (max. 285 mμ); vitamin A alcohol (max. 325 mμ); rehydro substance (max. 333 mμ, 348 mμ, and 369 mμ); oxidation product (max. 280 mμ); oxidation product (max. 280 mμ and below 235 mμ).

Effects of Addition of Water to the Isopropanol Samples.—Since the fatty acids had a stabilizing action on vitamin A palmitate in isopropanol, 5% of distilled water was added to the isopropanol to observe the effects of increased ionization of the fatty acids. The exact water content was 4.66% by Karl Fischer titration (4). This solvent will be referred to as "95%" isopropanol. The solutions of vitamin A esters were prepared and treated in the same fashion as the previous series except that vitamin A acetate was substituted for vitamin A palmitate because the latter was not sufficiently soluble in "95%" isopropanol.

A new assay method (6) was adopted because the "95%" isopropanol was not miscible with the cyclohexane of the B. P. method which is used as the diluting solvent.

$E_1 = 314$ mμ, $E_2 = 326$ mμ, $E_3 = 338$ mμ

$$E(1\%,1\text{cm.})\ (\text{corr.}) = \frac{3.61\ (2E_2 - E_1 - E_3)}{\%\ \text{dilution} \times \text{length of cell (cm.)}}$$

$$\text{Potency u./ml.} = E(1\%,1\text{cm.}) \times 1900$$

The rates were measured over a period of one hundred and twenty hours at which time the 45° samples showed 60% loss. The rates of degradation are given in Table II.

TABLE II.—RATE CONSTANTS FOR THE DEGRADATION OF VITAMIN A IN "95%" ISOPROPANOL SOLUTION

($k/2.303 \times$ hr.$^{-1} \times 10^4$)

	45°	37°	25°
Control (no fatty acid)	41.7	14.3	3.9
0.033 *N* Caproic acid	23.5	7.7	2.4
0.033 *N* Decanoic acid	24.5	10.1	4.1
0.033 *N* Palmitic acid	24.0	8.3	2.6
0.033 *N* Oleic acid	25.0	7.2	2.7
0.033 *N* Stearic acid	27.9	10.0	5.6

It appeared that in this case the fatty acids preserved the vitamin A potency and maintained a lower concentration of anhydrovitamin A, whereas water had a catalytic effect on vitamin A destruction.

A plot of log concentration *versus* time of the control series is submitted in Fig. 2.

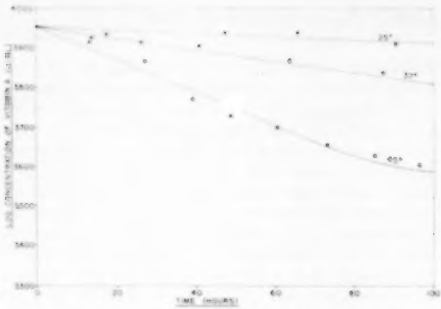


Fig. 2.—Vitamin A acetate in "95%" isopropanol.

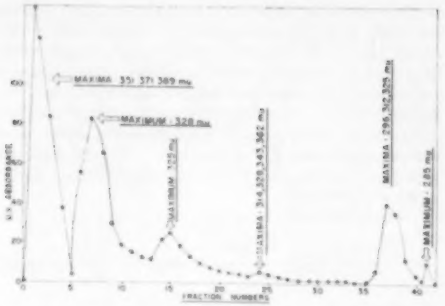

MAXIMA - 351, 371, 389 mμ
MAXIMUM - 328 mμ
MAXIMUM - 325 mμ
MAXIMA - 314, 328, 343, 362 mμ
MAXIMA - 296, 305, 325 mμ
MAXIMUM - 285 mμ
FRACTION NUMBERS

Fig. 3.—Chromatography of "95%" isopropanol control sample (45°).

Since it was shown that the extinction at 391 mμ could be used to determine the amount of anhydrovitamin A present (1) the following formula was derived to convert E_{391} mμ into the original units of vitamin A from which it was derived.

$$\frac{E_{391\ m\mu} \times 1115}{\text{per cent dilution} \times \text{length cell (cm.)}} = \text{units of vitamin A converted into anhydrovitamin A}$$

Calculations based on this formula showed that approximately 50% of the decomposition was of an eliminative nature.

Alumina Chromatography of the Samples.—The control, 0.033 *N* palmitic and caproic acid samples at 45° were chromatographed on alumina according to the procedure described under the alumina chromatography of "99%" isopropanol series.

Since many of the fractions contained identical substances it was not necessary to subject each fraction of U. V. and I. R. spectroscopy. A plot of U. V. absorbance *versus* fraction number gave a good indication as to when a new substance was being eluted. The center fraction of each group on the graph was chosen as the representative of the particular group as shown in Fig. 3. This fraction was subjected to U. V. and I. R. spectroscopy.

The following substances were eluted in the order mentioned: anhydrovitamin A (max. 351 mμ, 371 mμ, and 391 mμ); vitamin A ester (max. 326 mμ); esterified oxidation products (max. 290 mμ); rehydro structure (max. 328 mμ, 348 mμ, and 368 mμ); vitamin A alcohol (max. 325 mμ); oxidation products (max. 288–290 mμ).

Paper Chromatography.—The representative fractions were chromatographed on paper to determine if they were single substances or mixtures. Whatman filter paper No. 1 ($1^1/_2$-inch strips) were impregnated with 6% liquid petrolatum in petroleum ether (30–60°) and the solvent was allowed to evaporate. A drop of the sample was placed on the starting line and the strips were placed in a tank saturated with ethanol and nitrogen vapors. The strips were allowed to develop for seven to eight hours with ethanol U. S. P. as the eluant. The zones were identified by their fluorescence in U. V. light and by eluting the material from the paper with ethanol U. S. P. and determining their spectra. The controls had the following R_f values: vitamin A palmitate 0.090; acetate 0.514; and alcohol 0.895. Based on these R_f values all the samples contained vitamin A alcohol, ester (originally introduced), and oxidation products. The samples containing fatty acids also contained a small quantity of ester formed from the fatty acid of the media. The oxidation products had a higher R_f value than vitamin A alcohol (R_f 0.952); fixed oils, as olive oils, may also be used as an impregnating agent dissolved in petroleum ether 30–60°. Some of the alumina chromatographed fractions of oxidation products divided into two fractions. This indicated a degree of uncertainty of alumina separation of oxidation products. The vitamin A component of these solutions consisted of vitamin A alcohol, acetate, and an ester derived from the fatty acid of the media. This was significant because only vitamin A acetate was originally introduced and there was interconversion of vitamin A forms.

Action of Isopropanol, Water, Cyclohexane, and Fatty Acids on Anhydrovitamin A.—Embree and Shantz (7) and Oroshnick (8) prepared vitamin A active substances from the reaction of acetic, propionic, oleic, benzoic acids on anhydrovitamin A in ethanol or methanol. The products were the ethyl and methyl ethers of vitamin A. In nonalcoholic solvents the same reactants produced vitamin A esters (7).

In this work it was found that fatty acids decreased the loss of ester and also decreased anhydrovitamin A formation. It is possible that the stabilization was connected with a regeneration of a vitamin A substance by the fatty acids from anhydrovitamin A.

The solvents used in this study were cyclohexane, isopropanol (0.056% water), "95%" isopropanol (4.66% water), and "85%" isopropanol (14.36% water), as determined by the Karl Fischer titrations (4). BHT, 0.1%, was added to prevent oxidation of the vitamin A substance formed and it did not interfere with the U. V. assay of vitamin A. Cyclohexane was purified by distillation followed by chromatographing on completely activated alumina. It was transparent above 240 mμ. The 81–83° fraction from isopropanol distillation was used for this work. The anhydrovitamin A was prepared according to Shantz (2) by the action of HCl on vitamin A alcohol followed by purification on alumina deactivated by the addition of 3% water.

Calculation of Quantities of Vitamin A and Anhydrovitamin A.–A system was devised to determine the amount of vitamin A and anhydrovitamin

A present at any time "t." Since their U. V. spectra overlap, direct extinctions could not be used. The exact nature of vitamin A substance being formed in solution could not be determined; consequently an assumption was made that vitamin A alcohol was being formed. This was reasonable since the molar extinctions of vitamin A alcohol, ester, ether, and amine are practically equivalent. On the basis of this information, the following simultaneous equations were derived:

Anhydrovitamin A

$$E(1\%,1\text{cm.})_{351\ m\mu} = 2500, \quad E(1\%,1\text{cm.})\ 326\ m\mu = 700 \therefore \frac{A_{326}}{A_{351}} = 0.290$$

Vitamin A Alcohol

$$E(1\%,1\text{cm.})_{351\ m\mu} = 903, \quad E(1\%,1\text{cm.})\ 326\ m\mu = 1835 \therefore \frac{A_{326}}{A_{351}} = 2.032$$

Let x = extinction due to anhydrovitamin A at 351 mμ; Let y = extinction due to vitamin A at 351 mμ.

$$x + y = E_{351\ m\mu} \qquad \text{(Eq. 1)}$$

$$0.290\ x + 2.032y = E_{326\ m\mu} \qquad \text{(Eq. 2)}$$

solving

$$x = 1.166E_{351\ m\mu} - 0.574E_{326\ m\mu} \qquad \text{(Eq. 3)}$$

$$y = 0.574E_{326\ m\mu} - 0.166E_{351\ m\mu} \qquad \text{(Eq. 4)}$$

Cyclohexane Series.—In this series only caproic and acetic acids were studied and the samples were designed to contain 2, 4, and 6 M fatty acids in cyclohexane. The anhydrovitamin A was added to these solutions to make an approximate concentration of 0.1% (0.0037 M). Each sample was divided into three parts and placed in 60-ml. glass-stoppered bottles which were subsequently stored at 25, 37, and 50°. A control without fatty acids was also used. The bottles were flushed with dry nitrogen every time they were opened.

The samples were assayed by diluting 1 ml. of the reaction mixture to 100 ml. with isopropanol; then determining the extinction at 351 mμ and 326 mμ. Equations 3 and 4 above were used to calculate the extinctions at 351 mμ due to each substance.

Plotting the Data.—The experiments were designed so that the fatty acids would be in a large excess of the concentration of anhydrovitamin A, consequently the order of dependence on anhydrovitamin A could be determined from an appropriate plot of the data (9). Since anhydrovitamin A was converted into substances other than vitamin A, as evidenced by the precipitation of the polymerization products, a plot of log concentration anhydrovitamin A *versus* t would not give the true rate of vitamin A substance formation.

A theoretical calculation for the extinction at 351 mμ was made, based on the assumption that all the anhydrovitamin A was converted into a vitamin A substance. The value was called "A_∞" and was calculated as follows:

$$\frac{E(1\%,1\text{cm.})_{351\ m\mu} \times 286^1}{E(1\%,1\text{cm.})\ \text{Anhydrovitamin A}_{351\ m\mu} \times 268^1} = \frac{903 \times 286}{2500 \times 268} = 0.386 \qquad \text{(Eq. 5)}$$

[1] Molecular weights of the respective substances.

$$0.386 \times E_{351\ m\mu}\ \text{Anhydrovitamin A} = A_\infty \qquad \text{(Eq. 6)}$$

Another value A_t, the extinction at 351 mμ due to vitamin A substance formed at any time "t" was introduced. It was believed that a plot of log $(A_\infty - A_t)$ *versus* t would give the rate of anhydrovitamin A converted into vitamin A. This procedure helps correct for side reactions; although a small error is involved, it was considered negligible since only relative rates were desired. The plots were straight lines indicating a first order dependence on the concentration of anhydrovitamin A. No vitamin A was formed in the control without fatty acids present. These rates of conversion are found in Table III. Figure 4 represents the acetic acid samples at 50°.

TABLE III.—RATES OF VITAMIN A SUBSTANCE FORMATION OF ACETIC ACID AND CAPROIC ACID—ANHYDROVITAMIN A IN CYCLOHEXANE ($k/2.303 \times \text{hr.}^{-1} \times 10^4$)

Molarity	38°	50°
2 M acetic acid	1.25	2.60
4 M acetic acid	2.08	4.50
6 M acetic acid	3.90	7.50
2 M caproic acid	1.11	3.33
4 M caproic acid	2.37	5.85
6 M caproic acid	4.00	12.5

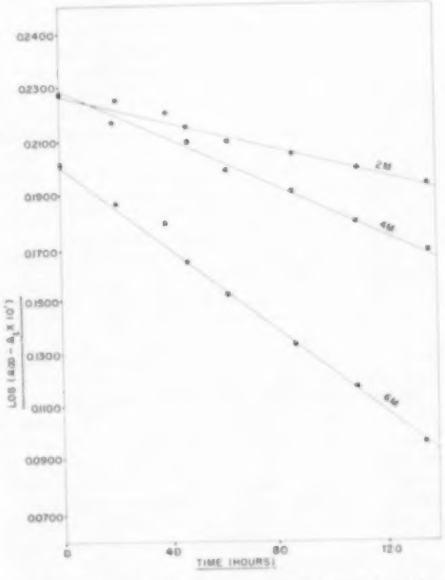


Fig. 4.—Rates of addition of anhydrovitamin A to acetic acid in cyclohexane at 50°.

Order of Dependence on the Fatty Acids.—A plot of log k *versus* log concentration of fatty acids produced lines with the following slopes: acetic acid at 50 and 37° = 1.05 and 1.09; caproic acid at 50 and 37° = 1.15 and 1.18. These data indicate a first-or-

TABLE IV.—RATES FOR TRANSFORMATION OF ANHYDROVITAMIN A INTO A VITAMIN A SUBSTANCE IN "99%" ISOPROPANOL

($k/2.303 \times hr.^{-1} \times 10^4$)

	"99%" Isopropanol Control	0.033 *N* Acetic Acid	0.033 *N* Caproic Acid	0.033 *N* Decanoic Acid	0.033 *N* Palmitic Acid
50°					
k_1	3.00	3.75	4.50	5.25	25.0
k_2	1.11	1.21	1.34	1.5	2.25
37°					
k_1	0.81	2.21	3.12	2.5	15.0
k_2	0.53	0.84	0.93	1.13	2.00

der dependence on the concentration of fatty acids in this solvent system.

Chromatography.—The cyclohexane solutions were extracted with aqueous NaOH to remove the fatty acids, and were subsequently dried with anhydrous Na_2SO_4. The solvent was removed *in vacuo* and the residue was dissolved in 5 ml. of petroleum ether 30–60°. This solution was chromatographed on alumina using the method described under the isopropanol-vitamin A palmitate series, to isolate the vitamin A active fraction from anhydrovitamin A and oxidation products. The ester followed anhydrovitamin A from the column. The solution of the ester was evaporated *in vacuo*. The concentrate was chromatographed on paper using the method described for the vitamin A acetate in "95%" isopropanol series. The R_f values for the controls were vitamin A acetate 0.643 and anhydrovitamin A 0.320. Both samples contained anhydrovitamin A and the respective ester formed from the fatty acid of the media. The caproate ester had an R_f value of 0.506.

Effects of Fatty Acids on Anhydrovitamin A in Isopropanol Solutions.—The action of fatty acids on anhydrovitamin A in isopropanol, "95%" and "85%" isopropanol was studied. The preparation of the samples was similar to the cyclohexane series except that isopropanol solvents were used and the following fatty acids: acetic, caproic, decanoic, and palmitic acids, were used, only in a 0.033 *N* concentration. Controls without fatty acids were also used. Table IV gives the rates of conversion using isopropanol as the solvent.

It appeared that the rate of conversion varied directly with the chain length of the fatty acid and that fatty acids catalyzed the conversion. The rates of conversion for the "95%" isopropanol systems are given in Table V.

TABLE V.—RATES FOR TRANSFORMATION OF ANHYDROVITAMIN A INTO A VITAMIN A SUBSTANCE IN "95%" ISOPROPANOL

($k/2.303 \times hr.^{-1} \times 10^4$)

"95%" Isopropanol Control	0.033 *N* Acetic Acid	0.033 *N* Caproic Acid	0.033 *N* Decanoic Acid	0.033 *N* Palmitic Acid
k 50°				
2.09	3.5	2.86	3.1	3.33
k 37°				
0.84	1.67	2.14	1.37	1.54
k 25°				
0.42	0.50	0.84	0.42	0.42

The addition of 5% water increased the reaction rates considerably (compare k_2 of Table IV), thus enabling the rate at 25° to be measurable.

The rates of conversion in the "85%" isopropanol series are given in Table VI. Figure 5 represents a plot of the "85%" isopropanol control.

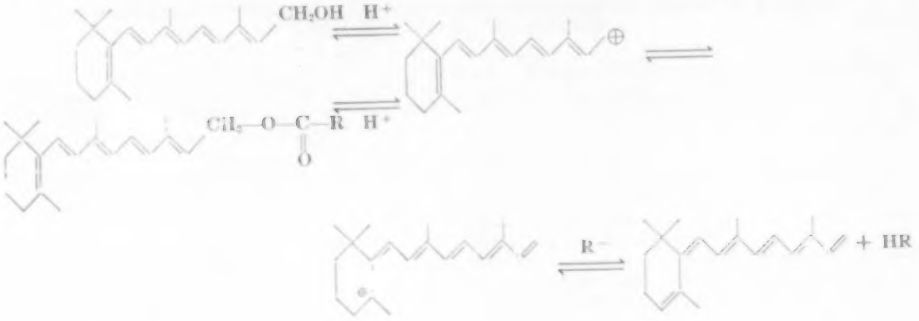
The rate appeared to vary directly with the increased water content and equilibrium was reached at one hundred and forty hours in the 50° samples. That the rates of the control, decanoic, and palmitic acid samples are almost equal could be explained by the fact that the latter two samples contained considerable amounts of oxidation products, giving the appearance of a slower rate of vitamin A substance formation.

Paper Chromatography.—The isopropanol systems were chromatographed on paper using the method described under "95%" isopropanol-vitamin A acetate systems. All three isopropanol systems had the same qualitative picture. The controls had the following R_f values: vitamin A palmitate, 0.070; decanoate, 0.200; caproate, 0.526; acetate, 0.630; alcohol, 0.930; and anhydrovitamin A, 0.370. All the systems contained vitamin A alcohol, oxidation products, and anhydrovitamin A. The fatty acid solutions, in addition, contained an ester formed from the fatty acid of the media. It was also observed that the ratio of vitamin A alcohol to ester increased as the per cent of water of the media increased.

DISCUSSION

Effects of Fatty Acids.—Caproic, decanoic, palmitic, stearic, and oleic acids demonstrated a stabilizing action on vitamin A esters in isopropanol when compared to a control without fatty acids. These data appeared to be different from the results obtained when vitamin A was dissolved in a vanishing cream base. These bases generally contain stearic acid, polyhydroxy alcohol, water, and an emulsifying agent, and the pH is usually on the basic side. Any one of these other ingredients could affect the destruction of vitamin A. The addition of 5% water to the isopropanol system increased the rate of elimination, but the fatty acid still retained some of its stabilizing action. The effects of water are in agreement with the results of Higuchi and Reinstein (1) who found that the rate of elimination of vitamin A acetate in hydroalcoholic media varied directly with the water content.

There were forms of vitamin A present in the isopropanol ester mixtures which were not originally

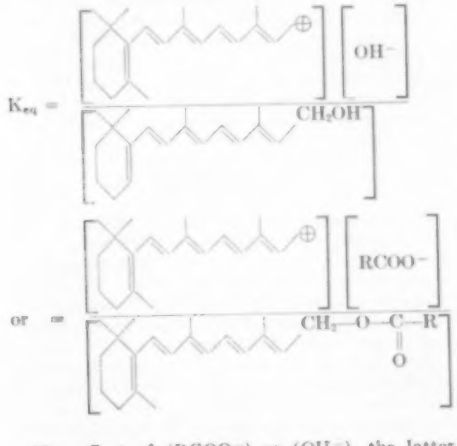


introduced as demonstrated by paper chromatography. This was believed to be due to a reaction of anhydrovitamin A with the fatty acid or water of the media.

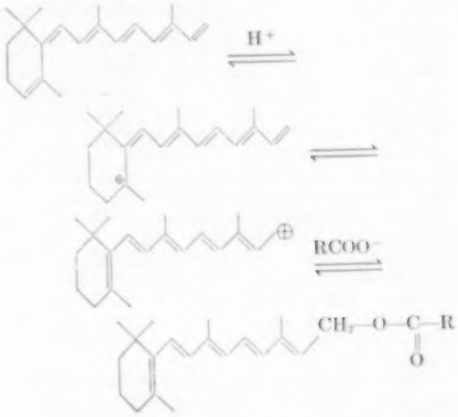
Action of Fatty Acids.—The control solutions without fatty acids invariably contained more anhydrovitamin A than the fatty acid solutions. Consequently, the increased stability of the esters in the presence of the fatty acids could be due to two reactions: (*a*) suppression of ionization of the ester, thus slowing down the rate of elimination, and (*b*) reaction of the fatty acid with anhydrovitamin A to reform a vitamin A substance.

It has been theorized by Embree (10) that the initial step of vitamin A alcohol degradation in the presence of acids is the formation of the carbonium ion. It is also possible that this is the first step with vitamin A esters (see equation above).

This initial step could be considered as an ionization represented by a typical equilibrium equation.



The effect of ($RCOO^-$) or (OH^-), the latter being known to prevent elimination of the acetate (1) can be seen from the law of mass action. Large quantities of these species can theoretically suppress ionization. The second suggested action of stabilization is possible since it is known that unsaturated compounds can undergo additional reactions with acids and also there are patents (7, 8) dealing with the conversion of anhydrovitamin A into vitamin A active substances in acid media. This reaction could be represented as follows:



This possibility was investigated by studying anhydrovitamin A in cyclohexane, isopropanol "95%," and "85%" isopropanol solutions of fatty acids. The fatty acids and water reacted with the substrate to give vitamin A esters and alcohol, respectively, which was always faster in the presence of the fatty acid.

The chromatographic analysis of the anhydrovitamin A-isopropanol systems showed that the "99%" isopropanol system contained vitamin A alcohol and ester as their active components; however, as the per cent of water increased, larger amounts of vitamin A alcohol were formed. Vitamin A treated with base will not eliminate (1), and the action of (H^+) on vitamin A can be terminated by the addition of base (2). Similarly, anhydrovitamin A treated with base will not reform a vitamin A substance. It was found in this and other work (7, 8) that fatty acids catalyze the formation of a vitamin A substance from anhydrovitamin A. This information supports the second suggested reaction of stabilization and that this "formation" reaction is only acid catalyzed.

Action of Water.—Water had a catalytic effect in both the forward and reverse reaction (destruction and formation of vitamin A). This catalytic action could be related to increased ionization of the reactants. Other hydroxylated solvents as methanol,

TABLE VI.—RATES FOR TRANSFORMATION OF ANHYDROVITAMIN A INTO A VITAMIN A SUBSTANCE IN "85%" ISOPROPANOL

($k/2.303 \times hr.^{-1} \times 10^4$)

"85%" Isopropanol Control	0.033 *N* Acetic Acid	0.033 *N* Caproic Acid	0.033 *N* Decanoic Acid	0.033 *N* Palmitic Acid
k (50°)				
4.55	8.00	5.72	4.94	4.45
k (37°)				
2.22	6.45	4.45	4.43	4.00
k (25°)				
0.863	1.11	0.874	1.6	1.56

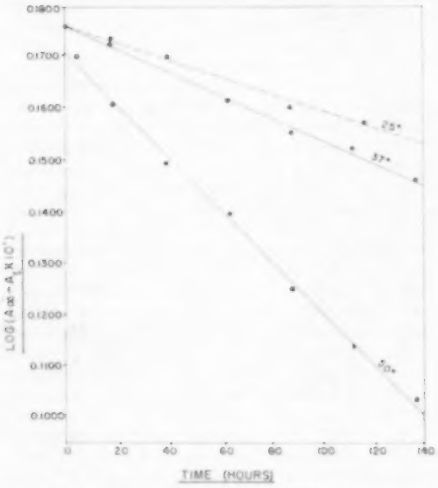


Fig. 5.—Anhydrovitamin A in "85%" isopropanol control.

ethanol, and isopropanol probably act by similar mechanisms but, due to lower polarity and solvating powers, they are less destructive.

Two rates, k_1 and k_2, were found in the "99%" isopropanol series regardless of whether vitamin A ester or anhydrovitamin A was the substrate (k_1 k_2). It appeared that k_1 and k_2 were related to the small water content (0.056%) of the media, because 95% and 85% isopropanol series only demonstrated one rate.

It would be logical to assume that k_1 is the rate due to water plus other factors. When the small amount of water was consumed, then k_2, which was slower, made its appearance. The fact that k_1 was faster in both directions resembles the catalytic effect of water in the "95%" and "85%" systems, which tends to confirm the original idea.

SUMMARY AND CONCLUSIONS

1. Vitamin A in hydrocarbon solvents as *n*-hexane solutions only showed oxidative degradation; in isopropanol, both oxidation and elimination had occurred.

2. The fatty acids in isopropanol solutions of vitamin A esters reduced the rate of vitamin A loss and maintained a lower concentration of anhydrovitamin A when compared to the control.

3. The addition of water to the isopropanol-vitamin A ester systems increased the rate of degradation and anhydrovitamin A formation. The fatty acids still maintained their stabilizing effect.

4. The reverse reactions, anhydrovitamin A plus isopropanol alone and with fatty acids showed that the fatty acid increased vitamin A formation when compared to a control.

5. Addition of water to systems mentioned in 4, increased rate of "vitamin A" substance formation.

6. In general, there does not appear to be any significant difference in the action of the different fatty acids studied.

7. The reaction of anhydrovitamin A plus fatty acid in cyclohexane was second order having a first-order dependence on each reactant.

8. The chromatographed fractions were identified by U. V. and I. R. spectra and R_f values.

9. The stabilizing action of the fatty acids appeared to be due to a regeneration of the ester in anhydrous solvents and a mixture of vitamin A alcohol and ester in hydroalcoholic media.

10. There appears to be a definite need for antioxidants in vitamin A solutions.

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Preparation and Stability of Some Esters of Vitamin A*

By ALBERT J. FORLANO† and LOYD E. HARRIS

Vitamin A esters of fatty acids having electronegative groups in the number 2 position were prepared. Acids, with chlorine or a double bond, were used and the esters were tested for stability against acid and solvolytic action. The results indicate that (*a*) stability against proton attack, in an anhydrous solvent, varies directly with the K_a of the acid portion, (*b*) the addition of water to an anhydrous solvent, containing HCl, decreases the rate of formation of anhydrovitamin A, and (*c*) stability in alcoholic solvents, without an acid catalyst, varies inversely with the K_a. A combined mechanism of vitamin A ester degradation in the presence of mineral acids and hydrolytic solvents is presented.

THE ACTION of lower alcohols and strong acids upon vitamin A and its esters to produce anhydrovitamin A is a well established phenomenon. Shantz, *et al.* (1), refluxed vitamin A esters in ethanol and found that a mixture of anhydrovitamin A, fatty acid, vitamin A ethyl ether, and other products were formed and that the addition of HCl to the ethanol catalyzed this reaction. Vitamin A alcohol is more sensitive to elimination in acid solution than the esters; however, in the absence of the catalyst, vitamin A alcohol is more stable (2). Prolonged contact of anhydrovitamin A with ethanolic HCl produces a substance called isoanhydrovitamin A (1), which is formed by the addition of a molecule of solvent to the double bond in the cyclohexene ring. Meunier (3) suggested a reasonable mechanism for (H^+) catalysis of anhydrovitamin A formation from vitamin A alcohol. No information has been found regarding the mechanism of proton catalyzed decomposition of the ester; however, the mechanism shown below appears to be satisfactory.

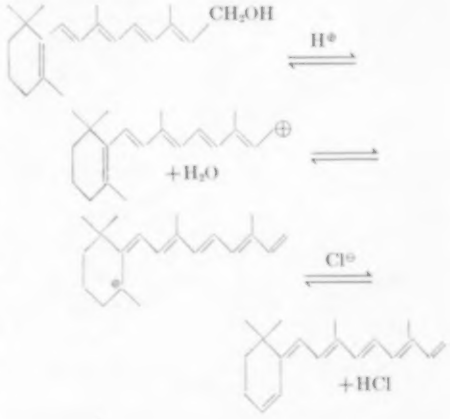

TABLE I.—$K_a \times 10^5$ OF ACIDS

Acetic acid	1.75
Chloroacetic acid	155.00
Dichloroacetic acid	5100.00
Trichloroacetic acid	120,000.00
Propionic acid	1.34
α-Chloropropionic acid	147.00
Acrylic acid	5.56
Butyric acid	1.48
Crotonic acid	2.03
Sorbic acid	1.73

Shantz, *et al.* (1), further substantiated the mechanism of ester decomposition when they found that anhydrovitamin A and fatty acid were formed when the ester was refluxed in ethanol. The greater stability of the ester in the presence of acid could be related to the electronegative effect of the carbonyl group on the alkyl oxygen. Reduction of the electron density around the alkyl oxygen should result in decreased attraction for protons, thus depressing the initial step of the reaction.

Based on this assumption, two series of esters were synthesized having electronegative groups in the 2 and 4 position of the fatty acid: (*a*) those containing chlorine in the α position as vitamin A chloroacetate and α-chloropropionate, and (*b*) those containing unsaturation in the 2 position as vitamin A acrylate, crotonate, and sorbate, the latter also having unsaturation in the 4 position.

Ingold (4) reported the K_a's of the acids pertinent to this study. These are listed in Table I.

* Received August 21, 1959, from the College of Pharmacy, The Ohio State University, Columbus 10.
Based, in part, upon a Ph.D. dissertation submitted to the Graduate School, The Ohio State University.
Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.
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The authors wish to express their gratitude to Mr. Paul Sleezer of Hoffmann-La Roche for his generous samples of vitamin A alcohol.

SYNTHESIS

Materials Used in This Study.—Vitamin A alcohol, Roche; chloroacetyl chloride, Eastman Organic (redistilled) b. p. 105–106°; trichloroacetyl chloride; Eastman Organic (redistilled) b. p. 114–116°; α-chloropropionyl chloride, Eastman Organic (redistilled) b. p. 109–111°; pyridine dried over $CaCl_2$ (redistilled) b. p. 114–115°; ethylene dichloride, Eastman Organic b. p. 82–84°; Woelm neutral activated alumina, Grade I for chromatography; petroleum ether b. p. 30–60°, Mallinckrodt (AR); quinoline, Eastman Organic b. p. 110–111°/14 mm.; N,N-dimethylaniline, Matheson, Coleman, and Bell b. p. 192–193.5°; sorbic acid, crotonyl chloride, and acrylyl chloride, Delta Chemical Co.

General Method of Preparation of Esters.—This method, a modification of the Baxter and Robeson method (5), was used for all the esters, except when modifications are specified.

Four and one-half grams (0.016 M) of vitamin A alcohol was dissolved in 25 ml. of ethylene dichloride containing 5 ml. of pyridine and cooled to 10° (I). In a separate flask, 0.018 M of the acid chloride was dissolved in 25 ml. ethylene dichloride (II). Solution II was added slowly to solution I with stirring and set in a dark place for two hours. The solvent was removed *in vacuo* and the residue was dissolved in 10 ml. of petroleum ether (30–60°). The instability of some of these esters necessitated their purification on alumina columns. The alumina was deactivated by the addition of 8% water in a glass-stoppered flask. After two hours of hydration, enough petroleum ether (30–60°) was added to the alumina to make a slurry which was poured into a glass column. Fifty grams of deactivated alumina were used for each Gm. of ester. The entire reaction mixture was poured on the column followed by petroleum ether (30–60°). Anhydrovitamin A was eluted first, followed by the ester. The former was distinguished from the latter by its orange color in U. V. light; the latter had a yellow-green color.

The ester fraction was evaporated *in vacuo* with the aid of a nitrogen bleeder, redissolved in petroleum ether, and rechromatographed as directed above. The residue was subjected to a high vacuum (0.5 mm.) for six hours at 35° to remove the pyridine or other bases. Quinoline and N,N-dimethylaniline were used in place of pyridine for the crotonate and acrylate esters, respectively. Attempts at crystallization from a variety of polar, nonpolar, and combination of both solvents were unsuccessful. The per cent yields are listed in Table II. Sorbic acid chloride was prepared by treating sorbic acid with thionyl chloride, s. a., and collecting the fraction that distilled at 79–80° and 13 mm.

The saponification equivalents of these esters were determined potentiometrically by the Schriner and Fuson method (7). The results are tabulated in Table II.

Infrared and U. V. Spectra.—The infrared spectra of these compounds were determined on a Baird infrared spectrophotometer, using sodium chloride plates. The spectra showed definite ester carbonyl peaks and the absence of hydroxyl peaks. The positions of the carbonyl peaks are tabulated in Table III. The $E(1\%,1cm.)$ values of the esters were determined in petroleum ether (30–60°) on a Beckman DU spectrophotometer at the U. V. maximum. The extinction coefficients and wavelength of the maxima are given in Table III.

TABLE III.—I. R. AND U. V. PHYSICAL CONSTANTS OF THESE ESTERS

Vitamin A Esters	Position Of I. R. Peak (μ)	E (1%,1cm.) (U. V.)	U. V. Maximum (mμ)
α-Chloropropionate	5.71	1300	328
Chloroacetate	5.64	1046[a]	325
Sorbate	5.80	1318	327
Acrylate	5.74	1387	327
Crotonate	5.76	1318	327
Palmitate	5.73	963	327

[a] This low extinction coefficient is an indication of its lack of stability.

The complete U. V. spectra were determined on a Cary recording spectrophotometer in petroleum ether (30–60°). All the esters, except the chloroacetate, had typical vitamin A spectra (maxima 325–328 mμ). The chloroacetate showed impurity peaks at 310 mμ, 341 mμ, and 360 mμ. Vitamin A sorbate had two peaks, one due to the chromophore of the vitamin A molecule, maximum at 327 mμ, and the second due to the sorbate part at 255 mμ.

Determination of R_f Values and Purity.—Using the system described in the previous paper (6), [6% liquid petrolatum in petroleum ether (30–60°)–ethanol, U. S. P.], the esters were chromatographed on paper as a means of identification and determination of purity. See Table IV.

The new esters with the exception of vitamin A chloroacetate, showed only one spot on the paper, indicating that they were pure. The chloroacetate appeared to be a mixtue of anhydrovitamin A and ester, indicating the sensitivity of the ester to ethanol.

TABLE II.—PER CENT YIELD AND SAPONIFICATION EQUIVALENTS OF THESE ESTERS

Vitamin A Esters	% Yield	Molecular Weight	Saponification Equivalent	% Deviation from Theoretical
α-Chloropropionate	93	376	385	+2.61
Chloroacetate	82	362	329	−9.13[a]
Trichloroacetate	[b]	...	...	...
Sorbate	80	381	385	+1.27
Crotonate	53	354	362	+2.21
Acrylate	45	340	347	+2.15

[a] The low saponification value is an indication of its poor stability.
[b] This ester could not be made under these conditions.

TABLE IV.—R_f VALUES OF VITAMIN A ESTERS AND RELATED COMPOUNDS

Compound	R_f Value	Compound	R_f Value
Neovitamin A alcohol	0.960	Vitamin A sorbate	0.570
Vitamin A alcohol	0.920	Vitamin A crotonate	0.550
Vitamin A methyl ether	0.725	Vitamin A chloroacetate	0.525
Vitamin A acetate	0.651	Vitamin A α-chloropropionate	0.515
Vitamin A acrylate	0.620	Vitamin A palmitate	0.100

Stability of These Esters.—The relative stability of these esters and related compounds toward eliminative degradation was determined in: (*a*) 0.01 *N* HCl in anhydrous ethanol [water content 0.148% determined by Karl Fischer method (8)]; and (*b*) 0.01 *N* HCl in ethanol U. S. P.

A sufficient quantity of material, such that the U. V. extinction at 326 mμ would be 0.700, was added to the acid solutions. These solutions were then transferred to silica cells and placed into a Beckman DU spectrophotometer and remained there through the entire determination. The temperature of the water flowing through the hydrogen lamp housing was maintained at 15 ± 1°. Anhydrovitamin A has an extinction at 326 mμ equal to 0.2 E_{328} mμ. The latter quantity was subtracted from the E_{328} observed to give a valid measurement of the vitamin A content at any time t. A plot of log ($E_{328\ \text{observed}} - 0.2\ E_{328}$) *vs.* t was linear. Since all the ingredients except vitamin A were in a large excess the rate was calculated from the slope of the line. The rates are given in Table V.

TABLE V.—RATES OF ELIMINATION OF VITAMIN A SUBSTANCES IN 0.01 *N* HCl IN ANHYDROUS ETHANOL[a] AND ETHANOL U. S. P.

Vitamin A Substance	$\frac{k}{2.303} \times 10^4$ min.$^{-1}$ (Anhydrous Ethanol)	$\frac{k}{2.303} \times 10^4$ min.$^{-1}$ (Ethanol U. S. P.)
Vitamin A chloroacetate	[b]	[b]
Vitamin A alcohol	150.0	6.60
Neovitamin A alcohol	65.0	..
Vitamin A methyl ether	23.0	1.15
Vitamin A sorbate	15.0	0.70
Vitamin A acetate	12.5	1.10
Vitamin A palmitate	9.62	0.30
Vitamin A crotonate	9.35	0.60
Vitamin A acrylate	4.77	0.20
Vitamin A α-chloropropionate	2.32	6.82

[a] Water content 0.148%. [b] Too fast to measure.

The esters were tested for stability against eliminative degradation in isopropanol and decanoic acid in isopropanol. The solvents were "99%" isopropanol and "95%" isopropanol [containing 0.056% and 4.66% water, respectively, as determined by the Karl Fischer method (8)], and a second series containing 0.033 *N* decanoic acid in both solvents. The solutions were prepared as described in the previous paper (6) under sections dealing with vitamin A esters in isopropanol and "95%" isopropanol. They were assayed by the method of Cama, *et al.* (9). The starting concentrations of these solutions ranged from 6000–8000 u./ml. The initial 45° rates of decomposition of the various esters are given in Table VI according to the method previously described (6) by plotting log concentration *vs.* time.

That the fatty acid did not appear to stabilize vitamin A chloroacetate in "95%" isopropanol is unexplainable at this time. The rates of the chloroacetate and α-chloropropionate degradation at 45° and the other temperatures indicate their extreme sensitivity to solvolysis. The 37° and 45° sample of the chloro esters reached equilibrium in five hours, whereas the other esters required twenty to thirty hours to equilibrate.

Chromatography of the Reaction Mixtures.—The reaction mixtures from the isopropanolic solutions were paper chromatographed, using the method described previously (6) under the section dealing with vitamin A acetate in "95%" isopropanol. The identification of the zones was based on their R_f values previously described and U. V. spectra in ethanol U. S. P.; vitamin A esters maxima 325–328 mμ; anhydrovitamin A 351, 371, and 391 mμ; and oxidation products 275, 290, and 310 mμ.

All the samples contained the ester originally introduced, vitamin A alcohol, and oxidation products. The acrylate, crotonate, and sorbate samples with fatty acids also contained small quantities of vitamin A decanoate, and larger amounts of vitamin A alcohol than the controls. The samples of the chloro esters with the fatty acids did not appear to contain vitamin A decanoate.

DISCUSSION

Mechanism of Decomposition of Vitamin A Alcohol and Esters.—The series of esters described in this paper was synthesized, based on the assumption that decreased electronegativity around the alkyl oxygen or carbonyl oxygen would decrease proton catalyzed attack.

When the stability of these esters was determined in "99%" and "95%" isopropanol, with and without a fatty acid present, their stability varied in this order:

Decreasing K_a of Acid Portion of Ester

Vitamin A trichloroacetate
Vitamin A chloroacetate
Vitamin A α-chloropropionate
Vitamin A acrylate
Vitamin A crotonate
Vitamin A sorbate
Vitamin A acetate
Vitamin A palmitate

Increasing Stability in Isopropanol

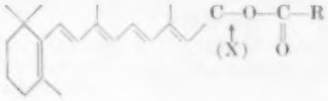
TABLE VI.—INITIAL DECOMPOSITION RATES OF VITAMIN A ESTERS IN ISOPROPANOLIC SOLUTIONS AT 45° ($k/2.303 \times 10^3$ hr.$^{-1}$)

Ester	"99%" Isopropanol	"95%" Isopropanol	0.033 *N* Decanoic Acid in "99%" Isopropanol	0.033 *N* Decanoic Acid in "95%" Isopropanol
Acrylate	7.50	22.50	5.00	10.00
Chloroacetate	85.00	43.20	59.0	83.00
Sorbate	4.16	5.00	1.66	3.22
α-Chloropropionate	56.10	83.50	50.0	80.00
Crotonate	5.75	6.66	3.13	4.55
Palmitate[a]	1.37	...	1.25	...
Acetate[a]	...	4.17	...	2.45

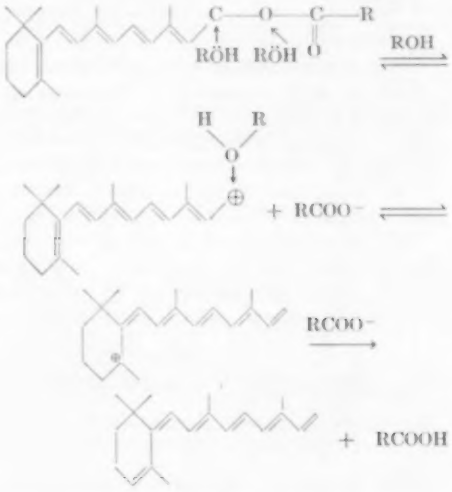
[a] Values derived from ref. (6).

Obviously there was a correlation between the K_a of the acid proton and decreased stability against elimination in isopropanol. The addition of 5% water to the anhydrous media catalyzed the elimination reaction which was to be expected.

It appeared that the electronegative group in the 2 position increased the K_a of the acid by reduction of electron density around the alkyl oxygen; this factor also resulted in a weakening of the covalent alkyl oxygen bond (X). This weakening rendered it more susceptible to solvolysis in hydroalcoholic



and alcoholic media as shown by the stability tests. This was further substantiated by the fact that little or no anhydrovitamin A was formed in hydrocarbon solvents. The reaction could be represented as follows:

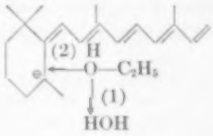


When the esters, vitamin A alcohol, neovitamin A alcohol, and methyl ether were treated with HCl in anhydrous ethanol, the stability was changed in this order:

Increasing K_a of Acid Portion of Ester		Increasing Stability in HCl-Anhydrous Ethanol
↓	Vitamin A chloroacetate[1]	←
	Vitamin A alcohol	
	Neovitamin A alcohol	
	Vitamin A methyl ether	
	Vitamin A sorbate	
	Vitamin A acetate	
	Vitamin A palmitate	
	Vitamin A crotonate	
	Vitamin A acrylate	
	Vitamin A α-chloropropionate	

The reason for the position of the chloroacetate can only be explained by the fact that the —C—O— bond is very sensitive to solvolysis in this compound, consequently, ethanol had sufficient solvolytic properties to destroy it rapidly. The stability of the other esters varied directly with the K_a of the acid radicals.

The degradation rates of these compounds in HCl-ethanol U. S. P. did not follow any definite pattern. Their rates of decomposition appeared to be a composite of proton attack and solvolysis; the acrylate ester was the most stable. This addition of 5% water to the anhydrous media reduced the rate of elimination about 10 times. This appears to be related to a competition of water molecules and the proton on the cyclohexene ring for the oxygen of the ethanol, thus reducing the basicity of the media and rate of elimination of the proton.

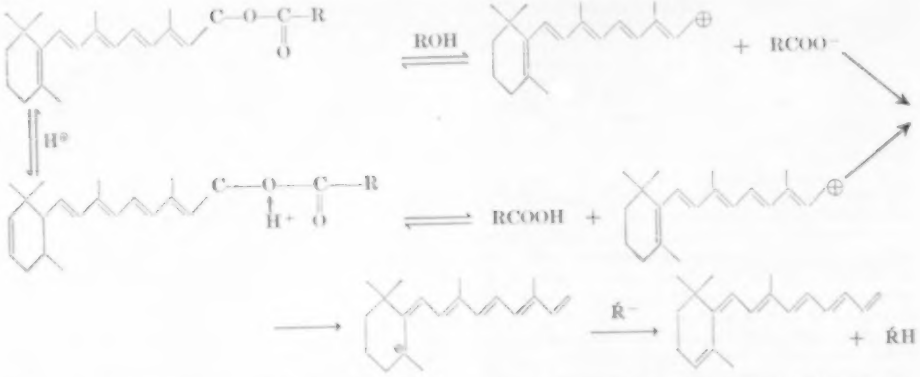


The differences in stability of these compounds in alcoholic solutions, with and without HCl suggested two mechanisms of decomposition.

(*a*) *Proton Catalyzed Attack.*—The esters containing fatty acids with large K_a's were attacked the least and this appeared to be related to the reduced electron density around the alkyl oxygen. Since the electronegative effect of hydrogen is less than that of a carbonyl group it is not surprising that vitamin A alcohol is more sensitive to the proton catalyzed attack than vitamin A esters.

(*b*) *Solvolytic Attack.*—In alcoholic or hydroalco-

[1] This compound is an exception to the rule.



holic media the stability against elimination is dependent on the strength of the carbon-oxygen bond (X). It has been observed that vitamin A alcohol is more stable than vitamin A esters in uncatalyzed solvent systems (2). It is reasonable to assume that the electronegative effect of the carbonyl group and the groups in the α position weakened the bond through an inductive effect on the electrons of the covalent bond. The electronegative effect of hydrogen (in vitamin A alcohol) is small compared to the strong electronegative groups mentioned. Solvolysis was further substantiated by Higuchi and Reinstein (2) who showed that pyridine in ethanolic vitamin A acetate solutions had no effect on the rate of elimination. Since pyridine would remove protons it appeared that the mechanism was solvolysis and not acid attack.

Stabilization by the one mechanism caused a corresponding decrease in stability by the other mechanism, therefore, it would appear most desirable to synthesize a molecule which is stable against solvolytic attack. This could possibly be done by the use of fatty acids with very low K_a values. The combined mechanism of decomposition is as shown above.

SUMMARY AND CONCLUSIONS

1. A method of synthesis for esters of vitamin A sensitive to water was developed.

2. The rate of eliminative degradation of vitamin A esters in isopropanol, aqueous-isopropanol, and 0.01 *N* HCl in ethanol was measured.

3. The physical constants for these new esters were determined.

4. The previous information on vitamin A alcohol, its esters, and the information in this study was correlated into the possible mechanisms of vitamin A decomposition.

5. Paper chromatography was used to determine the reaction products.

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An Investigation of Particle-Medium Interactions in Suspensions*

By W. A. HADDAD-LOUIS† and A. P. LEMBERGER

The physical significance of the two parameters as suggested by Robinson was investigated using Newtonian and non-Newtonian suspensions of glass spheres. System mean for each parameter did not vary significantly among the different suspensions. The initial viscosity and density of the medium seem to have no effect on the value of the parameters. An empirical equation used by Oliver can be rearranged when $k_1 = 0$, into a form similar to Robinson's equation. Since Robinson's equation can thus be viewed as a special case of the equation used by Oliver, it seems unlikely that the parameters have the physical significance assigned to them by Robinson.

RELATIVELY LITTLE is known about the influence of the dispersed phase on the overall rheological properties of suspensions, particularly at high volume concentrations of dispersed phase. Many empirical equations have been proposed which describe the rheological behavior of specific suspensions, but these equations cannot be made to apply to all systems.

Einstein derived a theoretically valid equation for the flow of simple systems under ideal conditions; however, the equation applies only at low volume concentrations of the dispersed phase. Several attempts have been made to extend Einstein's equation to higher volume concentrations of dispersed phase and to more complex systems. Some of these modified equations have been rather successful although they contain parameters without physical significance. Excellent reviews of the literature are given by Vand (1), Robinson (2, 3), and Ward (4).

Robinson (2, 3, 5) has proposed an equation with constants to which he has assigned physical significance. It was thought that equations which contain parameters to which physical significance can be attached could be usefully applied to a better understanding of the rheological properties of pharmaceutical suspensions and in a study of the contribution of the various components to the overall properties exhibited by a suspension. This study was undertaken to test the validity of the physical significance of the parameters in the equation proposed by Robinson.

THEORETICAL CONSIDERATIONS

The equation derived by Einstein for the flow of simple systems under ideal conditions states

$$\eta = \eta_o(1 + KV) \qquad \text{(Eq. 1)}$$

where η is the viscosity of the suspension, η_o is the viscosity of the dispersing medium, V is the volume fraction per unit volume of suspension of dispersed phase, and K is a constant with a value of 2.5 for spheres (3).

Equation 1 may be rearranged into the form

$$\eta_{sp} = KV \qquad \text{(Eq. 2)}$$

where η_{sp}, the specific viscosity of the suspension, is equal to $\eta/\eta_o - 1$.

In an attempt to extend the range of usefulness of Einstein's equation to higher volume concentrations, Robinson (2) proposed the equation

$$\eta_{sp} = KV/1 - S'V \qquad \text{(Eq. 3)}$$

where S' was defined as the relative sediment volume, the sediment volume per unit volume of solids. The quantity $1 - S'V$ then corresponds to the volume of free liquid. Robinson tentatively assigned a physical significance similar to a frictional coefficient to K; thus it would be influenced by such factors as a particle roughness, shape, and the presence or absence of an adsorbed shell of suspension medium around the particles. By plotting V/η_{sp} against V, the two parameters, K and S', are given by the reciprocals of the Y and X intercepts, respectively.

An empirical relation given by the equation

$$1 - \frac{1}{\eta_{rel}} = kV + k_1 \qquad \text{(Eq. 4)}$$

where η_{rel} is the relative viscosity (η/η_o) and k and k_1 are constants, was observed by Oliver (4, 6) to hold for suspensions. For stable suspensions $k_1 = O$.

It can be simply shown that when $k_1 = O$, Eq. 5 can be expressed as

$$\eta_{sp} = kV/1 - kV \qquad \text{(Eq. 5)}$$

which has the form of Robinson's equation. When $k_1 \neq O$, Eq. 4 can be rearranged to

$$\eta_{sp} = \frac{kV + k_1}{1 - kV - k_1} \qquad \text{(Eq. 6)}$$

From Eqs. 5 and 6 it appears that when $k_1 = O$, Robinson's K and S' should be equal to each other and identical with Oliver's k, while for suspensions where $k_1 \neq O$, the values of K and S' should differ due to the introduction of the constant k_1.

*Received August 21, 1959, from the University of Wisconsin, School of Pharmacy, Madison.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
This research project was supported in part by a grant from the Upjohn Co.
† Present address: Merck, Sharp and Dohme (I.A.) Corp., Cali, Colombia.

EXPERIMENTAL

Apparatus.—The Drage rheometer (7) was used for all the viscosity determinations in this study. The cup was immersed in a constant temperature bath set at 25.0 ± 0.1° during the determinations.

Materials.—Glass spheres (Minnesota Mining and Manufacturing Co.) were used as suspended material in all experiments. The spheres obtained were subjected to a fractional sedimentation in water to eliminate hollow spheres. Microscopic analysis performed on the spheres used gave a mean diameter of 30.08 μ, with a standard deviation of 3.94 μ. A periodic check on the density of the spheres during the course of the study showed a change from 2.438 to 2.475, indicating that the size distribution remained essentially constant.

Solutions of commercially available grades of methyl cellulose (Dow Chemical Co.), carboxymethyl cellulose (Hercules Powder Co.), acacia U. S. P., sucrose U. S. P., and strontium bromide N. F. were used as suspending media. Polyethylene glycol 400 (Carbide and Carbon Chemicals Co.), S. A. E. motor oils number 30 and 50 (Cities Service), and castor oil U. S. P. were also employed as suspending media.

Procedure.—The required amount of glass spheres was weighed to the nearest 0.1 Gm. and transferred to a calibrated Erlenmeyer flask. Some of the suspending medium was then added and mixed well with the glass spheres. Enough suspending medium was then added to dilute the suspensions to volume. The suspensions were then placed in a constant temperature water bath and allowed to come to temperature; about two hours was allowed.

Each suspension was then stirred well with a glass rod to break up any sediment which may have formed, the flask stoppered tightly, and allowed to tumble in a resuspending device for about ten minutes. The suspending medium was also treated in the same way. Enough of the suspension was then rapidly transferred to the proper cup and bob set, and the viscosity determined in the viscometer. The time elapsed between the transfer of the suspension to the cup and readings taken in the rheometer, both at increasing and decreasing rates of shear, was one to one and one-half minutes. Several up and down determinations were made. A centering device consisting of two half circles, with an opening in the center for the neck of the bob, was used to make sure the bob was centered. All runs were repeated three to four times except for the strontium bromide solutions where single determinations were made. Suspensions of the glass spheres were prepared on a weight-volume basis over a concentration range of 10–80% at 10% increments. Volume concentrations were calculated from the density of the glass spheres and suspension medium.

The glass spheres were recovered by repeated washings and decantations with distilled water. In some cases it was necessary to add a detergent to the water washings. In these cases, after washing, the glass spheres were allowed to stand overnight in chromic acid cleaning solution followed by repeated washing with distilled water until free of color. The spheres were then transferred to an evaporating dish and allowed to dry in an oven at 105° for ten hours.

RESULTS AND DISCUSSION

Calculation of Viscosities.—It has been found empirically that a plot of the logarithm of the rate of shear *vs.* the logarithm of the shearing stress yields a straight line, the slope of the line being dependent on the rheological behavior of the system. The equation of the straight line is

$$\log S = m \log \tau + \log a \qquad \text{(Eq. 7)}$$

which may be written

$$S = a\tau^m \qquad \text{(Eq. 8)}$$

where S is the rate of shear; τ is the shearing stress; $\log a$ is the intercept when $\log \tau$ equals zero, and m is the slope of the line (8–15).

The apparent viscosity, η_a, of the system at any point on the curve is given by the derivative of τ with respect to S: therefore from equation 7:

$$dS = am\tau^{(m-1)}\, d\tau \qquad \text{(Eq. 9)}$$

$$\frac{d\tau}{dS} = \frac{1}{ma\ \tau^{(m-1)}} \qquad \text{(Eq. 10)}$$

Equation 10 gives the viscosity of the system at any desired rate of shear, or shearing stress. When the substance behaves as a Newtonian liquid, $m = 1$ and Eq. 10 becomes:

$$\frac{d\tau}{dS} = \frac{1}{a} \qquad \text{(Eq. 11)}$$

where $1/a$ is the Newtonian viscosity. A convenient point to use as the basis of comparison for the viscosities of non-Newtonian substances is obtained by imposing the condition $\tau = 1$. Eq. 10 then becomes:

$$\frac{d\tau}{dS} = \frac{1}{ma} \qquad \text{(Eq. 12)}$$

The quantity $1/ma$ is the apparent viscosity of the system when $\tau = 1$, and is the viscosity used in this investigation for non-Newtonian fluids.

The slope, m, is obtained from any two points of the curve. The constant, a, can be obtained, by calculation, from the slope and a point on the curve. Equation 12 has been used by several workers (8, 9, 14, 15) as a basis of comparison for the rheological behavior of non-Newtonian systems.

In order to obtain experimental data to support the usefulness of this viscosity measure a series of experiments was designed with solutions of methyl cellulose 25 and 4000 grades, which show pseudoplastic behavior. Solutions of increasing concentration of each grade of methyl cellulose with viscosities ranging from 50 to 500 c. p. s. were tested for their viscosities according to Eq. 12 and the logarithms of the viscosities were plotted against the weight concentration of active ingredient. A straight line was obtained in each case. This behavior agrees with that obtained with other methods of viscosity determination.

Systems Studied.—Of the substances investigated, acacia and sucrose solutions, S. A. E. motor oils 30 and 50, and polyethylene glycol 400 showed Newtonian behavior, while solutions of methyl cellulose 25 and 4000 grades, sodium CMC medium viscosity, and strontium bromide in methyl cellulose showed psuedo-plastic behavior. The suspensions

of glass spheres in these substances behaved in the same way as did the original solutions of the substances.

Typical plots of Robinson's equation are shown in Figs. 1 and 2. It should be pointed out that the viscosities at the low solid concentrations yield points which are less reliable than those at high solid concentrations, since in the calculation two large figures are being subtracted to obtain a small number. The equation held well in the range of concentrations used. The parameters obtained from Robinson's plots, K and S', are summarized in Table I. It is to be noted that neither the viscosity nor the density of the original suspension seem to have any significant effect on the value

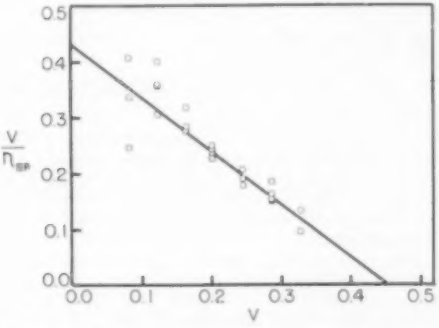

Fig. 1.—Robinson type plot of suspensions in CMC solution. $K = 2.27$, $S' = 2.17$.

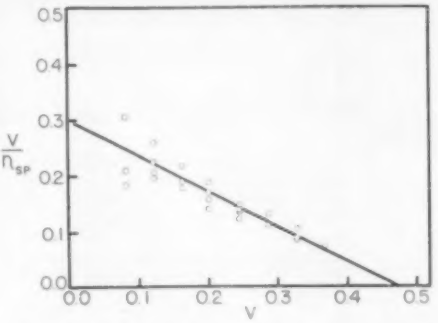

Fig. 2.—Robinson type plot of suspensions in methyl cellulose solution 4000 (initial viscosity = 227). $K = 3.33$, $S' = 2.11$.

of K. Robinson reported similar results from his investigations with the exception of the K values, which he found to vary depending upon the medium and which he postulated as being influenced by particle-medium interaction.

The difference in diameters of the particles used in this work compared to the 10–30 μ spheres used by Robinson is to be noted. If there is assumed to be an immobile liquid shell of constant thickness around each particle, then the smaller the particle the greater the relative effect this shell would have on the effective particle volume. This may account for the difference in values obtained by Robinson and those presently reported.

TABLE I.—PARAMETERS OBTAINED FROM ROBINSON'S PLOTS

Suspending Medium	Initial Viscosity, c. p. s.	Density		k	k_1	Constants		K (Calculated)
		Medium	Glass Spheres			K	S'	
Carboxy methyl cellulose (medium viscosity) soln.	188	1.0278	2.475	2.3	0.00	2.3	2.2	...
Castor oil	710	0.9602	2.468	2.3	0.00	2.4	2.3	...
Polyethylene glycol 400	88	1.1254	2.468	2.2	0.01	2.5	2.2	2.3
Sucrose solution	142	1.3270	2.468	2.3	0.01	2.7	2.3	2.4
Acacia solution	122	1.1246	2.470	2.0	0.11	3.0	2.3	2.8
Methyl cellulose 4000 soln.	133	1.004	2.438	2.0	0.10	2.8	2.1	2.9
Methyl cellulose 4000 soln.	227	1.004	2.438	2.0	0.15	3.3	2.1	3.5
Methyl cellulose 4000 soln.	507	1.004	2.438	2.1	0.07	3.0	2.1	2.8
Methyl cellulose 25 soln.	49	1.040	2.438	1.9	0.14	3.3	2.0	3.2
Methyl cellulose 25 soln.	220	1.040	2.438	1.9	0.09	3.2	1.8	2.9
Methyl cellulose 25 soln.	502	1.040	2.438	2.1	0.10	3.3	2.2	3.1
Strontium bromide in methyl cellulose 25 soln.	86	1.4818	2.468	1.8	0.15	3.2	1.9	3.2
Strontium bromide in methyl cellulose 25 soln.	108	1.5686	2.468	2.1	0.05	2.5	2.2	2.4
Strontium bromide in methyl cellulose 25 soln.	99	1.4545	2.468	2.0	0.08	2.7	2.1	2.6
Strontium bromide in methyl cellulose 25 soln.	98	1.2807	2.468	2.1	0.06	2.6	2.2	2.5
SAE No. 30 motor oil	211	0.8770	2.468	2.2	0.06	2.7	2.3	2.7
SAE No. 50 motor oil	555	0.8890	2.468	2.1	0.07	2.8	2.2	2.7

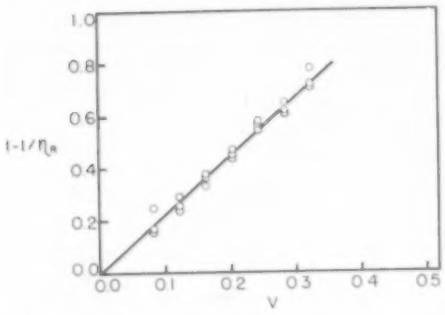

Fig. 3.—Oliver type plot of suspensions in CMC solution. $k = 2.26$, $k_1 = 0.00$.

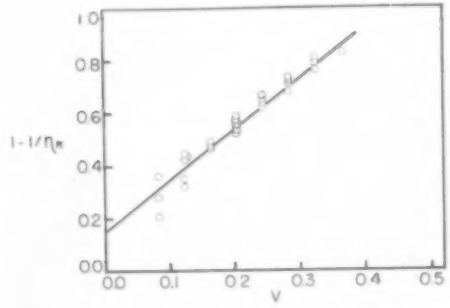

Fig. 4.—Oliver type plot of suspensions in methyl cellulose 4000 grade (initial viscosity = 227). $k = 1.95$, $k_1 = 0.15$.

The value of S', interpreted by Robinson as the relative sediment volume, showed a narrower variation range than did the values of K. The relative sediment volume per unit volume of glass spheres in water was obtained by multiplying the reciprocal of the bulk density by the true density of the glass spheres, thus:

$$1/1.41 \times 2.47 = 1.75$$

The bulk density was determined under the influence of gravity only. The difference in the values of the parameter S' determined experimentally and the relative packing volume of the glass spheres in water obtained here is significantly greater than that obtained by Robinson (2). If S' is the relative sediment volume, these results suggest that, in the media studied, a fairly thick layer of suspending medium is held immobile at the surface of the particles. This conclusion seems unlikely, however, in view of the small variation in S' observed with the different suspension media used in this study and the large size of the spheres used relative to molecular dimensions.

The data obtained in these studies have also been plotted according to the equation used by Oliver. Typical examples of these type plots are shown in Figs. 3 and 4. The values obtained for k and k_1 from the slope and intercept, respectively, are given in Table I. In the cases where $k_1 = O$, it can be observed that the values obtained for K and S' are close; they should in fact be equal. As k_1 increases from zero it can be seen that K and S' differ more and more due to the incorporation of k_1 into their values according to Eq. 6.

Since both Eqs. 3 and 6 seem to hold for the data obtained over the concentration range of suspended glass spheres used in this study, they may be equated.

$$\eta_{sp} = \frac{KV}{1 - S'V} = \frac{kV + k_1}{1 - kV - k_1}$$

and thus

$$K = \frac{(1 - S'V)(kV + k_1)}{V(1 - kV - k_1)} \qquad \text{(Eq. 13)}$$

If V is arbitrarily taken at some value at which both equations apply and the experimentally determined values for S', k, and k_1 are used, it is possible to calculate the theoretical value of K. These calculated values are given in Table I where V is taken at 0.25.

The agreement obtained between the calculated and the experimental values for K seems reasonable in consideration of the possible error in S', k, and k_1, which are determined experimentally. It would thus seem that Robinson's equation can be viewed as a special case of the empirical equation used by Oliver. If the physical significance of K is as a frictional coefficient, then it must be corrected for some other factor which appears as k_1 in Eq. 6. Indeed, since K and S' are expected to be numerically equal, it would seem that whatever physical significance they possess, if any, should be attributed to related properties.

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Accelerated Color Loss of Certified Dyes in the Presence of Nonionic Surfactants*

By MORTON W. SCOTT†, ALEX J. GOUDIE, and ARTHUR J. HUETTEMAN

The retention of color in aqueous and buffered solutions containing certified dyes (0.001 per cent) and nonionic surfactants (1.0 per cent) was measured after fourteen days storage at 48.8°. Six dyes, (FD&C Red No. 1, Blue No. 2, Red No. 4, Green No. 1, Yellow No. 5, and D&C Orange No. 3) and five commercially available surfactants (Tween 20, Igepal CA-710, Pluronic F-68, Myrj 52, and Brij 35) were included in the study. In all but four of the thirty systems examined, accelerated fading was noted over that obtained with solutions of the dye alone. For mixtures of FD&C Red No. 1 with Brij 35, and mixtures of FD&C Blue No. 2 with Pluronic F-68, the rate of fading was found to follow pseudo first order kinetics.

CONSIDERABLE EFFORT is often required in formulating color-stable pharmaceuticals containing certified dyes. While the use of antioxidants, chelating agents, and/or light absorbers may be of value in specific formulations, these additives do not exert protective effects in all systems. In the past, the formulating chemist also had at his disposal a reasonable number of certified dyes within each color category so that it was generally possible to select, after simple screening studies, at least one dye possessing satisfactory stability characteristics. Since the number of dyes on the certified lists now seems to be decreasing annually, the selection of a suitable dye is becoming more difficult.

Although the problem of maintaining color stability in pharmaceuticals and cosmetics is of general concern, little pertinent information on this problem has appeared in the literature. It is significant to note that quantitative data on the light and thermal stability of the certified dyes is not readily available in published reports. Some information can be obtained from general reviews released by the various dye manufacturers (1–3), but this data is only qualitative in nature. Details on the spectral characteristics and manufacturing techniques for several certified dyes can be found in the reports of Sclar and Freeman (4, 5). However, the interactions between dyes and other pharmaceutical reagents which may lead to poor color stability in finished products have received little attention.

Kuramoto, Lachman, and Cooper (6) have shown recently that reducing sugars, such as dextrose and lactose, have a deleterious effect on the color stability of FD&C Blue No. 2. While these results are not surprising in view of the known sensitivity of indigo dyes to reducing agents (7), the report does illustrate how common pharmaceutical materials may react with dyes to promote fading.

The present study was prompted by observations in our laboratories which suggested that nonionic surfactants may accelerate the rate of fading in products containing certified dyes. Since these surfactants are often used in colored preparations, it appeared worthwhile to explore this area in greater detail.

To simplify our experiments, it was decided to examine the color stability of dye-surfactant mixtures using simple aqueous solutions of the two components. For the initial survey experiments, six dyes, representing each of the major color categories, and five nonionic surfactants were selected. The surfactants included both ester and ether types which are widely used in pharmaceuticals. The dyes and surfactants were studied at 0.001% and 1.0%, respectively, because these compounds are commonly employed in similar concentrations in many formulations.

The systems containing FD&C Red No. 1 with Brij 35 and FD&C Blue No. 2 with Pluronic F-68 were taken for more detailed kinetic analysis. These systems were selected because they represented dye-surfactant combinations with widely differing properties and because their rates of color loss seemed most amenable to kinetic study.

EXPERIMENTAL

Materials.—Certified dyes used in this study included: FD&C Red No. 1, FD&C Blue No. 2, FD&C Red No. 4, FD&C Green No. 1, FD&C Yellow No. 5, and D&C Orange No. 3.[1] The commercially available nonionic surfactants employed for the study were: Tween 20,[2] Igepal CA-710,[3] Pluronic F-68,[4] Myrj 52,[5] and Brij 35.[6]

* Received August 21, 1959, from Johnson and Johnson Research, New Brunswick, N. J.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
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[1] Dyes obtained from H. Kohnstamm Co., Inc.
[2] Polyoxyethylene sorbitan monolaurate, Atlas Powder Co.
[3] Alkyl phenoxy polyoxyethylene ethanol, Antara Chemicals Division, General Dyestuff Corp.
[4] Condensate of polyoxypropylene with ethylene oxide, Wyandotte Chemicals Corp.
[5] Polyoxyethylene stearate, Atlas Powder Co.
[6] Polyoxyethylene lauryl ether, Atlas Powder Co.

TABLE I.—COLORIMETRIC DATA FOR DYE-SURFACTANT MIXTURES IN AQUEOUS SOLUTION

Dye-Surfactant Combinations[a]	Initial Observations		Observations After 14 Days at 48.8°	
	Absorbance[b]	Ratio of Sample to Control Absorbances	Absorbance[b]	Ratio of Sample to Control Absorbances
FD&C Red No. 1				
None	0.492	...	0.479	...
Tween 20	0.425	0.866	[c]	...
Igepal CA-710	0.430	0.875	0.287	0.600
Pluronic F-68	0.482	0.983	0.470	0.983
Myrj 52	0.443	0.902	0.000	0.000
Brij 35	0.439	0.893	0.094	0.196
FD&C Blue No. 2				
None	0.451	...	0.243	...
Tween 20	0.290	0.643	[c]	...
Igepal CA-710	0.415	0.922	0.000	0.000
Pluronic F-68	0.455	1.010	0.061	0.251
Myrj 52	0.430	0.955	0.007	0.003
Brij 35	0.305	0.698	0.000	0.000
FD&C Red No. 4				
None	0.452	...	0.420	...
Tween 20	0.388	0.858	[c]	...
Igepal CA-710	0.387	0.855	0.293	0.710
Pluronic F-68	0.437	0.968	0.403	0.960
Myrj 52	0.404	0.893	0.009	0.021
Brij 35	0.396	0.876	0.060	0.143
FD&C Green No. 1				
None	1.495	...	1.321	...
Tween 20	0.381	0.393	[c]	...
Igepal CA-710	1.031	0.690	0.140	0.095
Pluronic F-68	1.372	0.918	1.249	0.942
Myrj 52	1.459	0.975	0.019	0.007
Brij 35	1.495	1.000	0.052	0.025
FD&C Yellow No. 5				
None	0.504	...	0.491	...
Tween 20	0.504	1.000	[c]	...
Igepal CA-710	0.503	1.000	0.439	0.934
Pluronic F-68	0.499	0.992	0.080	0.163
Myrj 52	0.492	0.980	0.000	0.000
Brij 35	0.489	0.972	0.002	0.004
D&C Orange No. 3				
None	0.443	...	0.281	...
Tween 20	0.430	0.972	[c]	...
Igepal CA-710	0.421	0.952	0.157	0.558
Pluronic F-68	0.445	1.005	[c]	...
Myrj 52	0.438	0.990	0.049	0.174
Brij 35	0.433	0.978	0.159	0.566

[a] All dyes used at concentrations of 0.001% w/v; surfactant concentration at 1.0% w/v.
[b] Absorbance measured at wavelength of maximum absorption; FD&C Red No. 1 at 504 mμ, FD&C Blue No. 2 at 609 mμ, FD&C Red No. 4 at 503 mμ, FD&C Green No. 1 at 627 mμ, FD&C Yellow No. 5 at 413 mμ, D&C Orange No. 3 at 480 mμ.
[c] Sample was colorless and turbid.

Preparation of Samples for Survey.—Aqueous stock solutions of each dye and each surfactant were prepared at concentrations of 0.01% and 10.0%, respectively. In the case of the Pluronic F-68, Myrj 52, and Brij 35, mild heat and agitation were needed to speed dissolution of the surfactant. Aliquots of the stock solutions were blended and diluted with distilled water to give a series of 30 solutions, each containing a single dye at 0.001% and one surfactant at 1.0%. All possible 2-component combinations of the six dyes and five surfactants were prepared.

Samples were packaged in 50-ml. volumetric flasks (filled to the mark) and stored in the dark at 48.8 ± 0.5° for fourteen days. They were cooled quickly to room temperature, brought back to volume with distilled water if necessary, and analyzed. Control samples containing no surfactant and sample blanks containing no dye were handled in a similar fashion. A duplicate series of experiments was conducted in which M/15 Sorensen's phosphate buffer, pH 6.98, was used as the diluent for all stock solutions and samples.

Analysis.—The visible absorption spectra for the initial and aged samples were obtained with a Beckman model DK-1 recording spectrophotometer using 1.0-cm. Pyrex cells. pH values were determined with a Beckman Zeromatic pH meter.

Kinetic Studies.—Solutions containing 0.001% FD&C Red No. 1 combined with 1.0% Brij 35 and 0.001% FD&C Blue No. 2 combined with 1.0% Pluronic F-68 in M/15 Sorensen's phosphate buffer, pH 6.98, were prepared as outlined above. Aliquots of these solutions were stored at 21.1, 37.5, 48.8, 60.0, and 71.1° ± 0.5° for various time intervals. Control samples containing no surfactant were stored under the same conditions. At appropriate times, samples were removed from each temperature station for colorimetric analysis. A Beckman model DU spectrophotometer was used for all measurements. The samples were scanned occasionally on the model DK-1 instrument to

TABLE II.—COLORIMETRIC DATA FOR DYE-SURFACTANT MIXTURES IN M/15 SORENSEN'S PHOSPHATE BUFFER, pH 6.98[a]

Dye-Surfactant Combinations[b]	Initial Observations		Observations After 14 Days at 48.8°	
	Absorbance[c]	Ratio of Sample to Control Absorbances	Absorbance[c]	Ratio of Sample to Control Absorbances
FD&C Red No. 1				
None	0.444	...	0.441	...
Tween 20	0.391	0.881	0.003	0.007
Igepal CA-710	0.382	0.862	0.024	0.054
Pluronic F-68	0.430	0.970	0.410	0.930
Myrj 52	0.392	0.884	0.000	0.000
Brij 35	0.387	0.895	0.002	0.005
FD&C Blue No. 2				
None	0.410	...	0.333	...
Tween 20	0.410	1.000	0.139	0.416
Igepal CA-710	0.409	0.997	0.291	0.874
Pluronic F-68	0.414	1.008	0.226	0.679
Myrj 52	0.404	0.985	0.143	0.430
Brij 35	0.380	0.927	0.000	0.000
FD&C Red No. 4				
None	0.477	...	0.479	...
Tween 20	0.414	0.869	0.002	0.004
Igepal CA-710	0.399	0.837	0.198	0.413
Pluronic F-68	0.460	0.965	0.457	0.955
Myrj 52	0.407	0.853	[d]	...
Brij 35	0.400	0.840	0.000	0.000
FD&C Green No. 1				
None	0.999	...	0.978	...
Tween 20	0.057	0.057	0.058	0.057
Igepal CA-710	0.085	0.085	0.081	0.079
Pluronic F-68	0.843	0.844	0.776	0.823
Myrj 52	0.040	0.040	0.040	0.039
Brij 35	0.046	0.046	0.032	0.031
FD&C Yellow No. 5				
None	0.487	...	0.475	...
Tween 20	0.487	1.000	0.008	0.017
Igepal CA-710	0.477	0.979	0.214	0.451
Pluronic F-68	0.487	1.000	0.467	0.983
Myrj 52	0.473	0.971	0.008	0.017
Brij 35	0.479	0.982	0.002	0.004
D&C Orange No. 3				
None	0.410	...	0.407	...
Tween 20	0.393	0.958	0.000	0.000
Igepal CA-710	0.380	0.926	0.202	0.496
Pluronic F-68	0.403	0.985	0.396	0.973
Myrj 52	0.398	0.970	0.000	0.000
Brij 35	0.381	0.930	0.000	0.000

[a] All samples remained at pH 6.98 ± 0.02 over the storage interval.
[b] All dyes used at concentrations of 0.001% w/v; surfactant concentration at 1.0% w/v.
[c] Absorbance measured at wavelength of maximum absorption; FD&C Red No. 1 at 504 mμ, FD&C Blue No. 2 at 609 mμ, FD&C Red No. 4 at 503 mμ, FD&C Green No. 1 at 627 mμ, FD&C Yellow No. 5 at 413 mμ, D&C Orange No. 3 at 480 mμ.
[d] Sample was colorless and turbid.

determine if their wavelengths of maximum absorbance had shifted. In no case was such a shift detected. pH values were found to remain constant over the test interval.

RESULTS AND DISCUSSION

Table I summarizes the absorbance data for all unbuffered dye-surfactant combinations included in this study. The intensity of color remaining in the dye-surfactant samples compared to that remaining in the surfactant-free controls is also shown in Table I for both the initial and aged samples. This comparative retention of color has been expressed in terms of the ratio of the sample absorbance to that of the control, where both measurements were taken at the wavelength of maximum absorbance for the dye component alone. The selection of this wavelength for comparison was justified since it was found that in the presence of surfactant no wavelength shift occurred. Absorbance measurements were taken for all FD&C Red No. 1 systems at 504 mμ, Blue No. 2 at 609 mμ, Red No. 4 at 503 mμ, Green No. 1 at 627 mμ, and D&C Orange No. 3 systems at 480 mμ.

The results in Table I show that each of the surfactants tested exerts a deleterious effect on the color stability of the six certified dyes. After fourteen days aging at 48.8°, 26 of the 30 systems examined exhibited a comparative color loss greater thar 10%. In general, FD&C Blue No. 2 appeared most sensitive to the action of the surfactants, while Tween 20 and Myrj 52 produced the most dramatic effects on dye stability. In the systems containing Tween 20 and FD&C Blue No. 2 or Red No. 4, significant fading was noted immediately after preparation of the samples.

In all cases reported in Table I, a significant drop in sample pH from the initial values between 6.5–7.0 was obtained after aging. The final pH fell in the range of pH 3.5 to 5.0, although there was considerable variation between each surfactant system. Similar observations were obtained for the surfactant solutions containing no dye. In the case of the ester-type surfactants (Tween 20 and Myrj 52) this pH drop is believed to be the result of hydrolysis. This may, in turn, explain the appearance of turbidity in several of the Tween 20 samples. For the ether surfactants, the pH drop on aging is more difficult to explain. No indications of microbiological contamination were found in any of the fourteen-day samples.

In order to eliminate the possibility that dye fading was associated with the increase in acidity in aged samples, all dye-surfactant combinations were reprepared in buffer solution. The results of this study are shown in Table II. It is apparent that, while the pH has remained constant over the storage interval, accelerated fading still occurs. We have deliberately avoided making a comparison of the fading effects in the buffered and unbuffered solutions because different lots of surfactants were used for each study.

The exact interactions between dye and surfactant which lead to accelerated fading are not known. It would appear from the results in Tables I and II that some characteristic common to all of the surfactants is involved in the fading mechanism. Since all of the surfactants studied are ethylene oxide addition products, it seemed worthwhile to examine the effects of other ethylene oxide condensates on dye stability. Limited experiments using polyethylene glycol 200, polyethylene glycol 600, and polyethylene glycol 6000, showed that these materials also cause accelerated fading of the certified dyes. The reaction of dyes with surfactants may result therefore because of the presence of the ethylene oxide moiety in the surfactants. While we do not postulate a complexing phenomenon at this time, Waibel (8) has reported that (ethoxylated) nonionic surfactants exist in solution as polyoxonium compounds which actually are not nonionic, but weakly cationic. He states that these positively charged ions are capable of reacting with anionic coloring agents.

Figure 1 shows that the rate of fading of FD&C Red No. 1 in the presence of Brij 35 proceeds by two pseudo first order reactions.[7] The first reaction, which is comparatively slow, was observed at all temperatures. This was followed at temperatures above 48.8° by a more rapid second reaction. Table III presents the rate constants at the five temperatures studied.

It is important to note further that FD&C Red No. 1 in the absence of Brij 35 did not fade at any of the temperatures investigated. The accelerating effects of the surfactant, therefore, are clearly evident.

The Arrhenius plots for the Red-Brij system are shown in Figs. 2 and 3. These plots suggest that two mechanisms are involved in the fading reaction. At temperatures below 37.5° we find the first reaction which is characterized by an energy of activation of 21.9 Kcal./mole and a probability factor of 3.8×10^{-13}. At higher temperatures the second reaction occurs which has an energy of activation of 47.0 Kcal./mole and a probability factor of 1×10^{20}. These values are viewed with some reservation because replicate experiments showed that the kinetics may be altered significantly by what we believe to be lot to lot variations in the surfactant.

Figures 4 and 5 show that the rate of fading of FD&C Blue No. 2 in the absence and presence of Pluronic F-68 also follows pseudo first order kinetics. In this system, no induction period was obtained, although Kuramoto, *et al.*, have previously reported its presence (6). Table IV summarizes the rate data and Arrhenius plots for these systems are presented in Fig. 6.

It is apparent that in this particular study Pluronic F-68 produces a negligible effect on the color stability of the blue dye. In replicate studies,

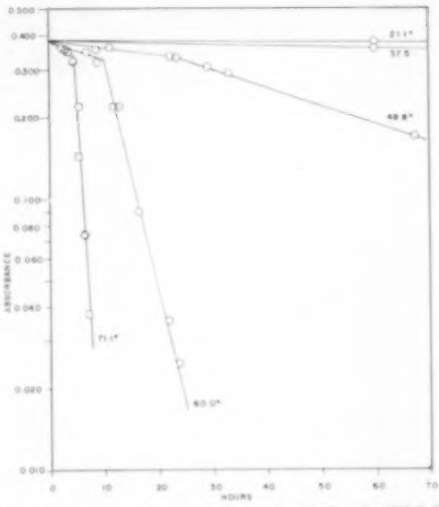

Fig. 1.—First order plots for color fading in FD&C Red No. 1–Brij 35 system.

TABLE III.—RATE CONSTANTS FOR THE FADING OF COLOR IN FD&C RED NO. 1-BRIJ 35 MIXTURES AT VARIOUS TEMPERATURES

Composition[a]	Temperature, °C., ±0.5° C.	Rate Constants (Reciprocal Hours)	
FD&C Red No. 1 (0.001%)	21.1	$<1.0 \times 10^{-5}$	
	37.5	$<1.0 \times 10^{-5}$	
	48.8	$<1.0 \times 10^{-5}$	
	60.0	$<1.0 \times 10^{-5}$	
	71.1	$<1.0 \times 10^{-5}$	
		First Reaction	Second Reaction
FD&C Red No. 1 (0.001%) plus Brij 35 (1.0%)	21.0	1.64×10^{-4}	...
	37.5	1.25×10^{-3}	...
	48.8	5.94×10^{-3}	1.53×10^{-1}
	60.0	1.51×10^{-2}	1.85×10^{-1}
	71.1	3.89×10^{-2}	8.09×10^{-1}

[a] Diluent: Sorensen's M/15 phosphate buffer, pH 6.98.

[7] At the lower temperatures, observations were extended considerably beyond that shown in Fig. 1.

Fig. 2.—Arrhenius plot for first fading reaction in FD&C Red No. 1–Brij 35 system.

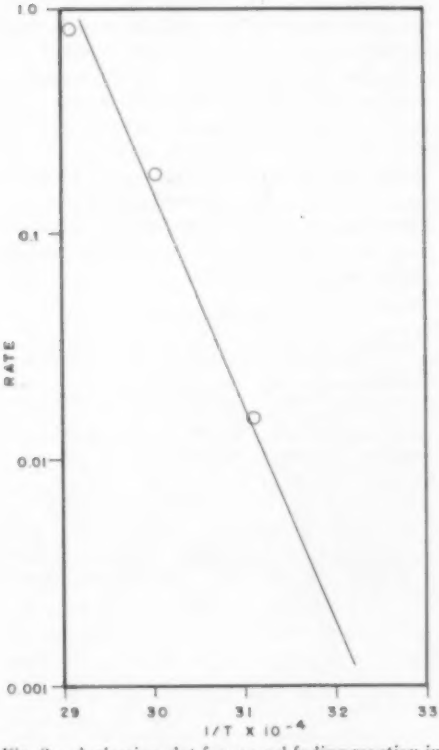

Fig. 3.—Arrhenius plot for second fading reaction in FD&C Red No. 1–Brij 35 system.

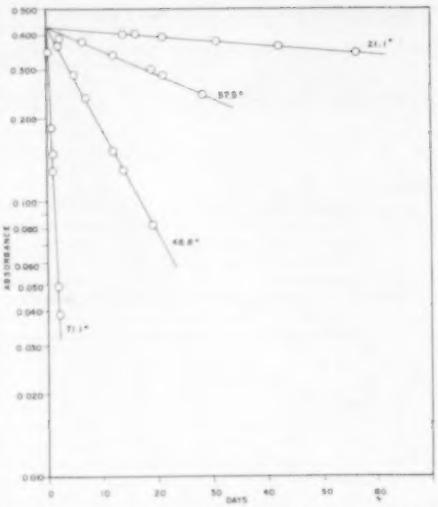

Fig. 4.—First order plots for color fading of FD&C Blue No. 2.

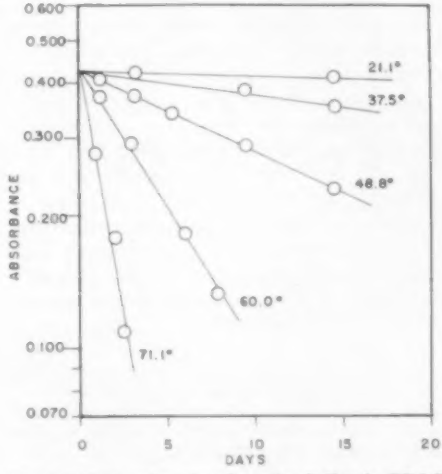

Fig. 5.—First order plots for color fading in FD&C Blue No. 2–Pluronic F-68 system.

we again noted that different kinetics may be obtained. For example, in one study the presence of Pluronic F-68 caused 227% increase in the rate of dye fading at 21.1° and an increase of 271% at 48.8°. These observations may be associated with the fact that different lots of surfactant showed different peroxide values and variable pH behavior when aged in aqueous solution. Kuramoto, *et al.*, have reported similar observations for the effects of sorbitol on FD&C Blue No. 2. They attributed these effects to the variable concentration of catalyst present in different lots of sorbitol.

Further experiments with the blue and red system

TABLE IV.—RATE CONSTANTS FOR THE FADING OF COLOR IN FD&C BLUE NO. 2-PLURONIC F-68 MIXTURES AT VARIOUS TEMPERATURES

Composition[a]	Temperature, ° C. ±0.5° C.	Rate Constants (Reciprocal Hours)	Energy of Activation	Probability Factor
FD&C Blue No. 2 (0.001%)	21.1	1.59×10^{-4}	22.4 Kcal./Mole	6.3×10^{13}
	37.5	7.99×10^{-4}		
	48.8	3.52×10^{-3}		
	60.0	...		
	71.1	4.18×10^{-2}		
FD&C Blue No. 2 (0.001%) plus Pluronic F-68 (1.0%)	21.1	8.57×10^{-4}	22.7 Kcal./Mole	6.3×10^{13}
	37.5	4.77×10^{-4}		
	48.8	1.95×10^{-3}		
	60.0	6.61×10^{-3}		
	71.1	2.09×10^{-2}		

[a] Diluent: Sorensen's M/15 phosphate buffer, pH 6.98.

Fig. 6.—Arrhenius plots for fading reaction in: (1) FD&C Blue No. 2–Pluronic F-68 system; (2) FD&C Blue No. 2 solution.

showed that the rate of color fading was not affected by preheating the surfactants before addition of the dye. These observations indicate that the mechanism of fading does not involve (stable) decomposition products of the surfactant.

CONCLUSIONS

The results of this study show that nonionic surfactants may have a deleterious effect on the color stability of certified dyes. The intensity of this effect has been found to vary from one dye-surfactant system to another and is not generally predictable. We believe further that variations in the lot to lot properties of surfactants may be an important factor in the color fading reactions.

While we have not attempted to postulate a mechanism for the fading reactions, our results suggest that interactions between the ethylene oxide moiety of the surfactant and the chromophoric groups of the certified dyes may be involved. This conclusion is confirmed in part by the fact that various polyethylene glycols also showed accelerating effects on the color fading of the certified dyes. We do not discount the possibility that trace contaminants which may appear in the surfactants and polyols as a result of the ethoxylation techniques are involved also.

In general, the degree of fading caused by nonionic surfactants in colored, multicomponent, pharmaceuticals and cosmetics will be less than that observed in the simple aqueous solutions studied in the present report. Our results do show, however, that interactions between surfactants and dyes should be carefully considered when color stability problems arise in products containing these materials.

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The Relationship Between Physiological Availability of Salicylates and Riboflavin and *In Vitro* Disintegration Time of Enteric Coated Tablets*

By A. B. MORRISON and J. A. CAMPBELL

Studies were conducted to relate the *in vitro* disintegration time of enteric coated tablets with physiological availability of salicylates and riboflavin, as determined by the urinary excretion of these drugs by human subjects. *In vitro* disintegration times were determined by the procedure given in U. S. P. XV Second Supplement, modified by the use of sixty minutes time in simulated gastric juice. Rates of urinary excretion of the drugs were determined after dosing subjects with seven salicylate preparations and five riboflavin preparations. Two products were found which disintegrated after less than thirty minutes in simulated gastric juice, while a third product was extremely resistant to the simulated digestive juices, and, in fecal recovery studies, was recovered intact from the feces. Studies on rate of urinary excretion of the drugs indicated delayed absorption from the enteric coated preparations. Salicylate tablets with *in vitro* disintegration times as long as two hundred and thirteen minutes were fully available *in vivo*, whereas a riboflavin preparation with an *in vitro* disintegration time of one hundred and twenty-eight minutes was only 41% available *in vivo*. It is suggested that enteric coated tablets should withstand the action of simulated gastric juice for at least sixty minutes, to ensure that these preparations do not disintegrate in the stomach. Furthermore, until quantitative *in vivo* data are available for individual drugs, it is suggested that to ensure full availability enteric coated preparations, other than salicylates, should disintegrate within thirty minutes in simulated intestinal juice.

ALTHOUGH ENTERIC COATINGS have been used on tablets for many years, surprisingly little work has been done to evaluate their effectiveness. Most reports on enteric coated preparations have described the composition of various coating mixtures, or have dealt with the results of *in vitro* disintegration tests. Little attention has been paid to *in vivo* availability in man. Kanig (1) reported that the *in vitro* disintegration times of enteric coated tablets taken from the same container varied from fifteen minutes to four hours. He concluded that "before enteric coatings may take their place among the reputable dosage forms, it is highly desirable that standardized testing procedures be instituted, in order to place the level of investigations in this field on a rational, easily duplicated, and thoroughly reliable basis." Crisafio, *et al.* (2), found that 31 of 64 samples of enteric coated preparations failed completely to resist the action of simulated gastric juice. Most of the products tested disintegrated within an hour in simulated intestinal juice. Trivedi (3) studied the disintegration of enteric coated tablets by use of an *in vitro* test similar to that given in U. S. P. XV (4) and observed wide variation in the disintegration time of tablets from sample to sample, and, in many instances, from tablet to tablet in the same sample. Tablet recovery from stools indicated a frequent loss of medication. Trivedi concluded that present U. S. P. standards are inadequate to rule out many faulty enteric coatings, and went so far as to suggest that enteric coatings might well be abandoned in favor of more reliable dosage forms.

Blythe, *et al.* (5), pointed out recently that *in vitro* tests alone cannot be used to predict the clinical effectiveness of enteric coated products. They stressed the need for studies on the relationship between *in vitro* data and quantitative *in vivo* results. Blood acetylsalicylic acid levels found with an enteric coated preparation were similar to those obtained with equivalent amounts of plain acetylsalicylic acid, indicating full availability of the medication *in vivo*.

Previous studies from this laboratory (6–9) showed that sugar coated tablets which did not disintegrate within sixty minutes (thirty minutes in simulated gastric fluid and the remainder of the time in simulated intestinal fluid) did not allow all of their contained drug to be released in human subjects, as measured by urinary excretion of riboflavin and *p*-aminosalicylate. The present experiments were conducted to study the relationship between *in vitro* disintegration time of enteric coated tablets and physiological availability of salicylates and riboflavin. Data were also obtained on variation in excretion rate over a period of time after dosing with the various preparations tested.

* Received October 2, 1959, from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario, Canada.

The authors are indebted to the subjects who took part in this investigation, and to Mr. C. Perusse for technical assistance.

METHODS

In Vitro Disintegration Times.—The apparatus and fluids described in U. S. P. XV (4) and the fluted disks described in U. S. P. XV Second Supplement (10) were used to determine disintegration time. The tablets were immersed in simulated gastric fluid for sixty minutes, and the remainder of the time in simulated intestinal fluid. Disintegration times reported were mean times of at least two separate tests on six tablets each, for tablets tested *in vivo* and *in vitro*, and of at least one test on six tablets for preparations tested only *in vitro*.

Physiological Availability.—Riboflavin or apparent salicylate levels were determined in the urine prior to dosing and after administration of standard or test preparations. Total salicylate was determined by a modification of the ferric nitrate method of Trinder (11), and riboflavin was determined by the U. S. P. fluorometric procedure (4). Studies were made on the stability of the salicylate in urine held under various conditions. The specificity of the method used for salicylate determination was checked by comparing the results found with those obtained by the method of Smith, *et al.* (12), which determines free salicylate, total salicylate, and salicyluric acid, separately. The amounts of salicylate or riboflavin obtained in the urine after dosing were corrected by subtracting the appropriate blank value determined on the urine of the same subjects. As in previous studies (6–9), physiological availability was calculated in the following manner

$$\text{Percentage physiological availability} = \frac{\text{Per cent excretion from preparation}}{\text{Percentage excretion from standard}} \times 100$$

Subjects.—Four to nine normal male subjects, ranging in age from 26 to 45 years, were used in the experiments. While on test, they continued to consume their regular diets, but were cautioned to eat meals similar in nature from day to day. During the riboflavin studies, the subjects refrained from eating foods high in riboflavin, such as liver.

Salicylate and Riboflavin Standards.—The subjects received doses of 10 gr. (648 mg.) or 15 gr. (972 mg.) of acetylsalicylic acid, or 5 mg. of riboflavin, in rapidly disintegrating standard tablets.

Commercial Preparations Tested.—Seven commercial enteric coated salicylate preparations, and five commercial enteric coated products containing riboflavin, were tested *in vivo*. In addition, six other salicylate preparations, and two other riboflavin preparations, were tested *in vitro*, but not *in vivo*. Salicylate preparations A, B, D, and J were samples from different lots of one product. Riboflavin preparations O and T were samples from different lots of the same product, as were preparations P and R. Each subject received from 10 to 20 gr. (648 to 1,296 mg.) of salicylate, or 3 mg. of riboflavin, from the various preparations. All products were assayed for salicylate or riboflavin prior to administration.

Assay Periods.—The standards or commercial preparations were given at 8 : 45 a. m. after breakfast. Urine was collected in opaque bottles after two, four, six, eight, fourteen, and twenty-four hours, and at four-hour intervals thereafter until the rate of excretion approached blank values. For the riboflavin studies, 2 ml. of 3.5 N H_2SO_4 was added to each bottle, to ensure stability of the vitamin.

Fecal Recovery Studies.—Three subjects were each given 10 tablets (6.5 Gm.) of salicylate preparation C. The feces were collected for up to seventy hours after ingestion of the tablets. Undissolved tablets were separated from the feces by gently brushing the stool sample through a screen.

RESULTS

Salicylate Studies.—Studies on the validity of the method used for salicylate determination showed that the values found for total salicylate by a modification of the method of Trinder (11), were in good agreement with those obtained by the procedure of Smith, *et al.* (12). The latter method utilizes an ethylene dichloride extraction and determines free salicylate, total salicylate, and salicyluric acid, separately. Since the modified Trinder method is easily carried out on large numbers of urine samples, it was used in the present experiments. The stability of salicylate in urine was studied by collecting urine from a subject dosed with 975 mg. acetylsalicylic acid and storing the urine overnight at room temperature, or in the refrigerator. Aliquots of the sample held at room temperature were treated by layering with toluene or were acidified with 3% of 3.5 N H_2SO_4. The values found for acetylsalicylic acid (determined as salicylate) in mg. per ml. of urine were as follows: fresh urine, 1.11; refrigerated urine, 1.04; urine held at room temperature, 1.08; acidified urine held at room temperature, 1.02; toluene treated urine, 1.03. Since the results showed salicylate in urine to be stable at room temperature overnight, it was not felt necessary to refrigerate the samples or to acidify them.

Descriptive data on the salicylate preparations used are summarized in Table I. Of the products tested *in vivo*, two contained sodium salicylate, while the remainder contained acetylsalicylic acid. The *in vitro* disintegration times of the salicylate products varied from twenty-seven minutes (product G) to over four hundred and eighty minutes (product C). As is indicated from the disintegration time, product G was unable to withstand the action of gastric fluid. Product C, on the other hand, was extremely resistant to digestion. Some preparations (e. g., products G and K) showed wide variation in *in vitro* disintegration time of tablets taken from the same container, whereas other products (e. g., products A, B, N) showed little variation from tablet to tablet.

The data on urinary excretion of acetylsalicylic acid (measured as salicylate) after ingestion of 650 mg. or 975 mg. of the standard dose are summarized in Table II. The mean excretion in four trials varied from 74.6 to 89.2%, with an overall mean of 84.4%. The latter value agrees well with results on salicylate excretion reported in the literature (13). The mean percentage excretion in trial 1 (975 mg. dose) was less than that in the other three trials (650-mg. dose). The decrease is not believed to be related to dosage level, however, since the results were not consistent

TABLE I.—DESCRIPTION OF ENTERIC COATED SALICYLATES STUDIED

Product	Medication	*In Vitro* Disintegration Time, min.	Salicylate per Unit, gr.	Salicylate per dose, gr.
A	Acetylsalicylic acid	87 ± 5.0[a]	5.0	15.0
B	Acetylsalicylic acid	86 ± 4.4	5.0	15.0
C	Acetylsalicylic acid	480 ± ...	10.0	20.0
D	Acetylsalicylic acid	110 ± 27.1	5.0	15.0
E	Sodium salicylate	213 ± 21.3	10.0	10.0
F	Sodium salicylate, *p*-aminobenzoic acid, ascorbic acid	145 ± 8.9	5.0	15.0
G	Acetylsalicylic acid, ascorbic acid	27 ± 23.1	10.0	10.0
H	Acetylsalicylic acid	75 ± 4.0	5.0	...
J	Acetylsalicylic acid	111 ± 22.7	5.0	...
K	Acetylsalicylic acid	76 ± 30.3	5.0	...
L	Salicylic acid, *p*-aminobenzoic acid	100 ± 16.1	3.75	...
M	Sodium salicylate, *p*-aminobenzoic acid, ascorbic acid	100 ± 10.8	5.0	...
N	Salicylic acid, *p*-aminobenzoic acid	117 ± 5.9	3.75	...

[a] Standard deviation.

TABLE II.—PERCENTAGE OF STANDARD DOSE OF ACETYLSALICYLIC ACID RECOVERED IN URINE

Subject	Trial 1 15-gr. dose	Trial 2 10-gr. dose	Trial 3 10-gr. dose	Trial 4 10-gr. dose
ABM	84.1	78.4	81.6	...
JAC	59.2	...	...	78.1
CP	87.8	95.4	...	90.0
MM	67.5	89.6	93.4	92.5
CR	70.7	93.6	92.2	88.5
TKM	96.2	100.2	99.1	96.2
KS	59.3	87.5	70.7	...
TP	78.1	86.9	...	...
DS	68.3	60.4	98.5	77.4
Mean	74.6	86.5	89.2	87.1

TABLE III.—PHYSIOLOGICAL AVAILABILITY OF SALICYLATE IN SEVEN PRODUCTS[a]

Subject	Per Cent Salicylate Available in Product— A	B	C	D	E	F	G
ABM	51	89	0	111	84	...	110
JAC	107	106	..	131	124	133	...
CP	85	80	26	94	...	94	119
MM	102	112	4	...	112	...	107
CR	76	92	0	91	112	99	136
TKM	88	73	2	105	123	101	130
KS	95	108	90	57	119	104	123
DS	93	100	41	114	136	112	84
TP	...	...	..	109	...	106	...
Mean	87	95	23	101	116	107	116
In Vitro disintegration time, min.	87	86	480+	110	213	145	27

[a] Products A, B, and D were samples from different lots of the same product.

from subject to subject; some subjects (e. g., ABM, CP) showed relatively constant percentage excretion from the standard dose in repeated trials, whereas others were more variable. Because of the intersubject and intertrial variation in percentage excretion, the average of all trials was used in calculating physiological availability on each subject.

The average blank excretion of apparent salicylate in the urine without dosing varied from 0.10 mg. to 0.16 mg. per minute at the various collection periods.

The results of the physiological availability studies with the salicylate preparations are summarized in Table III. Excretion values for products containing sodium salicylate were expressed in terms of acetylsalicylic acid. Considerable variation was noted between subjects. Despite the wide range in *in vitro* disintegration times (Table I), all of the preparations except preparation C, were available physiologically. The latter preparation, which had an *in vitro* disintegration time exceeding four hundred and eighty minutes, was only 23% available on the average. This value is somewhat misleading, since the product was essentially unavailable to four of the seven subjects who consumed it. Subject KS, however, showed a good availability of salicylate in this product (90%), illustrating the great variation encountered between subjects. Most of the subjects showed apparent availabilities of somewhat over 100% for the salicylate in products E, F, and G. Such variability is well known in biological assays.

Data on mean rate of excretion of salicylate after ingestion of the seven products were compared with those for the combined standards in Fig. 1. As suggested by Swintosky, *et al.* (14), a semilog plot was used. None of the preparations showed a peak excretion rate as high as the standard, although all except product C were fully available physiologically. The peak of salicylate excretion occurred earlier with the standard than with any of the preparations studied. With the exception of product C, the excretion of salicylate was more sustained with the products tested than with the standard. Products A and B, which were two samples of the same preparation, showed peak net excretion rates of 0.57 and 0.48 mg./min. respectively.

Product C, which showed low availability, also showed low and nonsustained urinary excretion. The curve shown is actually somewhat misleading, since it represents average values, and the salicylate in the preparation was essentially unavailable to four of seven subjects. Prior to eight hours after ingestion of product C, there was no detectable net urinary excretion of salicylate. The rate of excretion found with product D declined at approximately the same rate as the standard, but the peak of excretion occurred later. Product E, which disintegrated slowly *in vitro* (two hundred and thirteen minutes), showed a peak excretion rate later than did the other products studied. The rate of excretion found with this product declined much

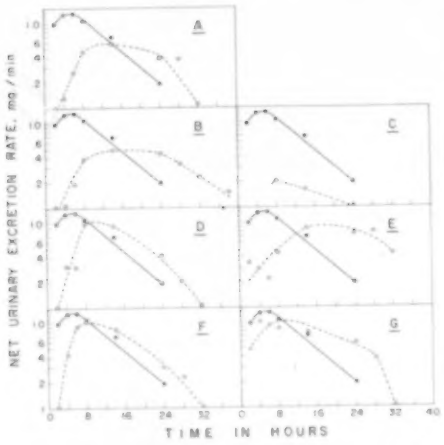

Fig. 1.—Urinary excretion curves of salicylate in seven tablets O–O–O–, compared with curve for a salicylate standard tablet ●—●.

more slowly than did that with the standard. Products F and G both showed peak excretion rates of approximately 1 mg./min. Although product G disintegrated *in vitro* after only twenty-six minutes in gastric juice, its peak excretion rate did not occur earlier than those of the other preparations.

The fecal recovery studies with salicylate product C showed that all three individuals who consumed 10 tablets of this preparation recovered the tablets in the feces. These results agree with those obtained by urinary excretion measurements, which also showed poor availability of this product. Tablets were recovered in as little as twelve hours after ingestion in one individual, and as late as seventy hours after ingestion in a second subject. A few of the recovered tablets showed cracks in the enteric coat, but all were intact, with only the sugar coat removed. In a few of the tablets which had passed through the gastrointestinal tract, the enteric coat was removed by hand. The core of the tablets was still extremely hard, and even in the absence of the enteric coat, would be expected to resist disintegration for some time.

Riboflavin Studies.—Descriptive data on the riboflavin-containing preparations studied are summarized in Table IV. *In vitro* disintegration times varied from twenty-four minutes (product V) to two hundred and forty-one minutes (product P). As with the salicylate products some of the riboflavin preparations (e. g. products P, T) showed wide variation in *in vitro* disintegration time of tablets from the same container, whereas other products were less variable. In all cases, the dose of riboflavin administered was 3.0 mg. Although it perhaps might have been desirable to increase the dose to the level of the standard, 5 mg., it was not convenient to do so, since most of the preparations contained high levels of iron. Product V, which disintegrated in gastric juice, could not be termed enteric coated.

Data on the percentage excretion of the standard dose of riboflavin were presented previously (9), and those for blank excretion values were published elsewhere (15). Briefly, the mean excretion, in 5 trials, varied from 57 to 60%, with an overall mean of 58%.

The results of the physiological availability studies with the riboflavin preparations are summarized in Table V. In contrast to the results obtained with the salicylate preparations (Table III), only two of the riboflavin products had availability approaching 100%. Product O, which had an *in vitro* disintegration time of one hundred and four minutes, was 81% available *in vivo*. Preparation S, which disintegrated in eighty minutes *in vitro*, showed an average *in vivo* availability of only 75% in two trials. Products P, R, and T, which had disintegration times of two hundred and forty-one, one hundred and twenty-eight, and two hundred and forty-seven minutes were only 29, 41, and 40% available, respectively. The high variation noted between subjects with all products is typical of preparations on the borderline of *in vivo* availability (6, 9).

Data on rate of excretion of riboflavin after ingestion of the five products were compared with those for the standard in Fig. 2, using a semilog plot. Since only 3 mg. of each of the riboflavin preparations were ingested, the rate values calculated were multiplied by 1.67 to make them comparable to the 5 mg. standard. The peak urinary excretion rate was directly related to physiological availability, and occurred earlier with the standard than with the products tested. Products O and S, which showed the highest physiological availability, also showed the highest peak excretion rates. Products P, R, and T, which were poorly available *in vivo*, showed peak excretion rates only 17 to 29% that of the standard. With the possible exception of product P, the rate of riboflavin excretion declined somewhat more slowly with the preparations tested than with the standard.

DISCUSSION

The results of the present studies showed that tablets labeled as enteric coated exhibited widely differing *in vitro* disintegration times and physiological availabilities. Two preparations were found which disintegrated *in vitro* in simulated gastric juice. These products do not conform to any acceptable definition of enteric coated products, despite label claims. On the other hand, tablets of salicylate product C were extremely resistant to disintegration and were recovered intact from the feces. It is obvious that this product would not exert the desired therapeutic effects. These findings would indicate that a significant proportion of commercially available enteric coated salicylates and multivitamin-mineral preparations do not satisfactorily fulfill the purpose for which they were intended.

It is interesting to note the difference in the *in vivo-in vitro* relationship found with salicylate and that found with riboflavin. Salicylate preparations with *in vitro* disintegration times as long as two hundred and thirteen minutes were fully available *in vivo*, whereas a riboflavin preparation with an *in vitro* disintegration time of one hundred and twenty-eight minutes was only 41% available *in vivo*. This difference may be due, in part, to the fact that salicylates are readily absorbed well down in the intestinal tract (13), whereas there is evidence that

TABLE IV.—DESCRIPTION OF ENTERIC COATED VITAMIN PREPARATIONS STUDIED

Product	Medication	*In Vitro* Disintegration Time, min.	Riboflavin per Unit, mg.	Riboflavin per Dose, mg.
O	Multivitamins and minerals	104 ± 9.7[a]	1.0	3.0
P	Homatropine, caffeine, vitamins, and ammonium chloride	241 ± 58.6	1.0	3.0
R	Homatropine, caffeine, vitamins, and ammonium chloride	128 ± 7.2	1.0	3.0
S	Multivitamins and minerals	80 ± 6.4	1.0	3.0
T	Multivitamins and minerals	247 ± 35.6	1.0	3.0
U	Multivitamins and minerals	114 ± 8.5	2.5	...
V	Multivitamins and minerals	24 ± 3.3	1.0	...

[a] Standard deviation.

TABLE V.—PHYSIOLOGICAL AVAILABILITY OF RIBOFLAVIN IN FIVE PRODUCTS[a]

Subject	Per Cent Riboflavin Available in Product O	P	R	S[b]	T
ABM	97	0	23	33	20
JAC	113	44	29	80	24
CP	..	30	..	..	..
MM	85	..	69	..	..
TKM	73	..	12	109	72
KS	36	37	66	78	45
DS	..	33	45	..	..
Mean	81	29	41	75	40
In Vitro disintegration time, min.	104	241	128	80	247

[a] Products O and T were samples from different lots of the same product, as were products P and R.
[b] Mean of two trials.

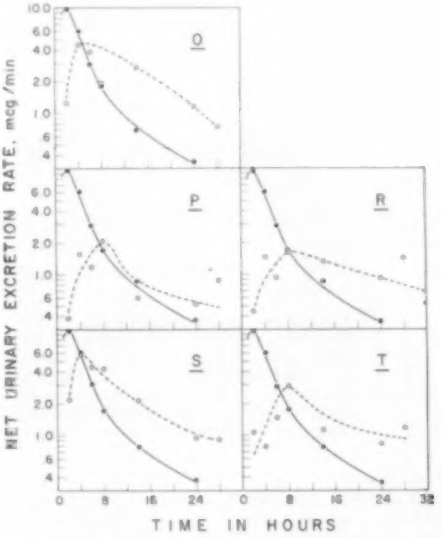

Fig. 2.—Urinary excretion curves for riboflavin in five tablets O–O–O–O, compared with curve for a riboflavin standard tablet ●—●.

riboflavin is destroyed in the lower portion of the intestine (16).

The variation between subjects in percentage physiological availability of the preparations tested was probably a reflection, in part, of the well-known variation in clinical response of patients to doses of drugs. It was probably influenced also by the variation in disintegration time of tablets taken from the same container. For some products, the variation in *in vitro* disintegration time between tablets was very large, indicating the need for improved production control procedures. It is of interest to note that the subjects used by Blythe, *et al.* (5), showed considerable variation in blood salicylate levels, probably, in part, due to the factors mentioned above.

On the question of time limits for *in vitro* disintegration of enteric coated tablets, it is obvious that all products should withstand the action of simulated gastric juice for at least sixty minutes. In fact, Blythe, *et al.* (5), recommended that enteric coated tablets should resist attack by simulated gastric fluid for at least four hours.

Although it is difficult, from the data available, to propose exact time limits for the disintegration of enteric coated salicylate preparations in simulated intestinal juice, there seems to be no reason why such products should not disintegrate within about four hours. On the other hand, it appears that enteric coated riboflavin preparations must disintegrate within about thirty minutes in simulated intestinal juice, to show full physiological availability. Thus, when tested *in vitro* by the procedure used in the present studies, enteric coated vitamin preparations containing riboflavin should disintegrate within ninety minutes (sixty minutes in simulated gastric juice and thirty minutes in simulated intestinal juice). This time limit would correlate well with earlier work on riboflavin (6, 8, 9), in which it was found that, to be fully available, sugar coated tablets must disintegrate within sixty minutes (thirty minutes in simulated gastric juice and the remainder of the time in simulated intestinal juice).

Further information is required on the relationship between *in vivo* availability and *in vitro* disintegration time for other enteric coated preparations. Until such information is available, however, it would seem advisable to suggest that enteric coated preparations (other than salicylates) should disintegrate in simulated intestinal juice within the time limit suggested for riboflavin, i. e. thirty minutes. This proposal may need to be modified as quantitative *in vivo* information becomes available for specific drugs.

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Emulsification With Ultrasonic Waves I*

Evaluation of the Minisonic and a Modified Analysis Procedure

By ROBERT E. SINGISER† and HAROLD M. BEAL

A modified photomicrography procedure has been developed to speed the size-frequency method of analyzing emulsions. This method employs a film strip and rule simultaneously projected. Classification of 1,000 oil globules into the proper size intervals can be done in about one-half of the time formerly required. The Minisonic homogenizer was investigated for (*a*) the effect of long insonation periods on emulsions and (*b*) various methods of manufacturing the emulsion. No coagulation of emulsions occurred when exposed to ultrasonic waves for thirty minutes.

RECENTLY ultrasonic equipment operating on the "liquid whistle" principle has been made commercially available. This type of apparatus, for the first time, makes practical ultrasonic emulsification in production quantities. The laboratory size model of this apparatus, the Minisonic,[1] was evaluated in this investigation. The effect of prolonged exposure of emulsions to high frequency sound and the optimum method of producing the emulsion were studied.

Unless simple gross observations are to be relied upon, a size-frequency analysis is necessary to evaluate the emulsion. From the analysis data, numerous parameters may be calculated. A simpler, faster method for a size-frequency analysis of emulsions has been perfected.

* Received August 21, 1959, from Abbott Laboratories, Scientific Division, North Chicago, Ill.

This paper is based on a dissertation submitted by Robert E. Singiser to the Graduate School of the University of Connecticut in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

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[1] Sonic Engineering Corp., 146 Selleck St., Stamford, Conn.

ANALYSIS TECHNIQUES

In a size-frequency analysis at least 1,000 internal phase globules are usually measured. This has varied from 50 to 50,000, with most investigators using the 1,000–4,000 range. Statistically, the reliability of the results varies with the square root of the number of counts made. Harkins and Beeman (1) carried out the first size-frequency analysis on emulsions in 1929. A microscopic mounting of the emulsion was projected onto a screen; then the individual globule images were traced and measured. Others have determined globule diameters from photomicrographs (2) or by projecting the microscopic field onto drawing paper with a camera lucida attachment; then tracing the globule outlines (3, 4). These procedures were reported to require a minimum of one to one and one-half hours to measure 1,000 globules. Often an assistant is required. With the exception of the photomicrographic procedure no permanent record was obtained.

The method of analysis used in this study was quicker and easier than those previously reported. A permanent record was also obtained.

Equipment.—A Helber counting chamber and standard hemocytometer cells were used to mount emulsions for microscopic examination. In all of the cells the central portion is ruled into squares

0.0025 mm.² in area (50 × 50 μ). A Bausch and Lomb microscope having a self-contained lighting source was employed, utilizing the 43× high power lens. The light intensity could be controlled with a diaphragm and/or a transformer.

A Leitz Micro-Ibso attachment with a 35-mm. Leica camera was placed on the microscope. This setup permits quick and accurate photomicrography. It is possible to prepare, mount, and photograph a large number of emulsions per day and to analyze the emulsions at a more convenient time.

The emulsions were diluted sufficiently for easy counting, generally from 1:100 to 1:450. The diluted emulsion was allowed to stand for thirty to forty-five minutes so that the dispersed globules would rise to a single plane. Photographs of three to four fields on the ruled cell were taken.

After the film was developed, it was projected directly on a screen via a strip film projector. At the same time, a horizontal rule was projected. The rule was composed of vertical lines, the distance between each representing 5 μ when projected. The rule was permanently mounted in the projector. This arrangement allowed the rule and film frame to be in focus simultaneously. The usual magnification was approximately 5,000 diameters. The horizontal rule was standardized against a photograph of the Helber cell where the width of the squares is known to be 50 μ.

Measuring Technique.—In the analysis of the globules, the exact diameters were not recorded. Class limits of 1 μ were selected, and the oil globule diameters were recorded as frequency of occurrence within the class limits. It was found that despite the final magnification of 5,000× the optics of the microscope-camera device precluded differentiating between globules less than 1 μ and those of 1–2 μ. Therefore, the first two class intervals had to be combined into one 0–2 μ class interval. Any globules greater than 10 μ in diameter were included in the 10–11 μ class and arbitrarily assigned an average diameter of 10.5 μ.

The film strip was passed down over the rule, and each globule was measured as it fell on the rule. Measurements were made on only a small vertical strip of the film at each pass over the rule. The measurements were recorded on an eight key blood cell calculator. Each key represented a class interval (e. g., 0–2, 2–3, 3–4 μ, etc.). As a globule was measured, it was recorded by depressing the properly designated key. Each key totaled individually. It was not necessary to look away from the screen to record the measurement; appreciably speeding up the analysis. As ten class intervals were used it was necessary to record by hand two of the less frequently used intervals. Measurements of 1,000 or more globules could easily be made in twenty-five to forty-five minutes, depending on the dispersion within the sample. This effected a considerable saving in time.

Reproducibility.—The reproducibility of the method of analysis was checked by making three complete measurements on two different emulsions. One emulsion was very uniform in dispersion, the second emulsion quite coarse with globules distributed throughout the counting range. The results of the triplicate measurements are given in Table I. The mean globule diameter (d_{as}), standard deviation (s), and specific interfacial area (S) were calculated.

Minisonic Homogenizer

The Minisonic apparatus is a hydrodynamic device which operates on the Pohlmann "liquid whistle" principle. It has the advantages over other ultrasonic generators of: (*a*) having a continuous flow, (*b*) operating directly in the liquid with little energy loss, and (*c*) using the relatively low frequency of 22 kc. Alexander (5) believes oxidation and depolymerization reactions are held to a minimum at low frequencies.

The Minisonic has a single or double funnel arrangement which holds the emulsion components and acts as a reservoir for the gear pump. Heat of 60–70° is generated in the liquid if it is recycled for long periods. To avoid this cavitational heating, a water-cooled jacket was constructed around the outer funnel. This arrangement permitted recycling for thirty minutes while maintaining the temperature at 35°.

Methods of Manufacture Studied

The manufacturer of the Minisonic suggests that emulsions be prepared using a two-funnel method where the continuous phase is placed in the outer funnel and recycled while the disperse phase is fed into it from the inner funnel. Oil-soluble and water-soluble additives are to be added in their respective phases.

The manufacturer's suggested method for making emulsions was compared with the method of Atlas Powder Co. (6) where both hydrophilic and lipophilic agents are added to the oil phase. Otherwise the two-funnel procedure was used.

A third method of preparation was also evaluated. This consisted of preparing coarse stock emulsions or premixes by hand shaking in a gallon bottle. Aliquots were then withdrawn and run in the Minisonic with the inner funnel removed.

Emulsions Evaluated.—Oil-in-water emulsions of 25% mineral, peanut, and safflower oils, each using a Span 80[2]/Tween 40[2] emulsifier system, were prepared by each of the three methods of manufacture. The total Span-Tween concentration was 4%, the relative amounts of each used was calculated from their respective HLB values of 4.3 and 15.6 for o/w emulsions of mineral oil (HLB 12), peanut oil (HLB 8), and safflower oil (HLB 11.5).

Similar emulsions of each oil were made with a Span 80/Pluronic F-68[3] emulsifier system of 4% total concentration. The relative amounts used were based on respective HLB values of 4.3 and 16.[4] The Atlas method of manufacture could not be evaluated in this instance since the Pluronic F-68 was not dispersible in the oil phase.

Evaluation.—The emulsions were insonated in the Minisonic homogenizer for fifteen minutes. Following this a size-frequency analysis was done as described. From these data the mean globule diameter (d_{as}), its standard deviation (s), and the specific interfacial area (S) were calculated. The results are shown in Table II.

[2] Product of Atlas Powder Co., Wilmington, Del.
[3] Product of Wyandotte Chemical Co., Midland, Mich.
[4] HLB values experimentally determined for safflower oil and Pluronic F-68, other values from the literature.

TABLE I.—TRIPLICATE MEASUREMENTS

	Peanut Oil/Acacia Emulsion			Safflower Oil/Pluronic-Span Emulsion		
Trial	1	2	3	1	2	3
N[a]	1,168	1,203	1,305	1,081	1,098	1,213
d_{ao}[b]	1.71 μ	1.63	1.85	3.09	3.15	3.20
s[c]	0.86 μ	0.82	0.86	2.43	2.43	2.48
S[d]	2.39×10^4	2.49×10^4	2.35×10^4	0.90×10^4	0.91×10^4	0.90×10^4

[a] N, Total number of counts. [b] d_{ao}, arithmetic mean globule diameter. [c] s, standard deviation: $s = \sqrt{\frac{n(\Sigma fx^2) - (\Sigma fx)^2}{n(n-1)}}$
[d] S, specific interfacial area, cm.^{2}/ml.

TABLE II.—EFFECT OF THE VARIOUS METHODS OF INCORPORATING EMULSION COMPONENTS IN THE MINISONIC

Diameter, μ	Premix Single Funnel	O-S/Inner Funnel[a] W-T/Outer Funnel[b]	O-S-T/Inner Funnel[c] Water/Outer Funnel	Premix Single Funnel	O-S/Inner Funnel[a] W-P/Outer Funnel[d]
	—Mineral Oil-Tween 40/Span 80 Emulsion—			—Mineral Oil-Pluronic F-68/—Span 80 Emulsion	
1.0	70.75[e]	84.53	78.64	69.57	52.66
2.5	21.28	9.70	12.39	22.06	21.60
3.5	5.01	4.42	6.05	7.79	14.65
4.5	1.07	0.48	0.66	0.58	3.47
5.5	0.36	0.10	0.47	...	2.54
6.5	0.36	0.19	0.85	...	2.16
7.5	0.45	0.48	...	...	1.41
8.5	0.09	0.10	0.47	...	0.85
9.5	...	...	0.09	...	0.38
10.5	0.09	...	0.38	...	0.28
	100.00	100.00	100.00	100.00	100.00
N[f]	1,118	1,041	1,057	1,206	1,065
S[g]	1.74×10^4[j]	1.91×10^4	1.56×10^4	2.35×10^4	1.18×10^4
d_{ao}[h]	1.57[k]	1.33	1.51	1.55	2.26
s[i]	1.05[k]	0.88	1.23	0.87	1.73
	—Peanut Oil-Tween 40/Span 80 Emulsion—			—Peanut Oil-Pluronic F-68/—Span 80 Emulsion	
1.0	76.08	71.13	73.33	84.43	84.47
2.5	21.45	13.06	14.30	11.19	8.94
3.5	2.12	9.79	9.20	3.73	4.97
4.5	0.35	2.84	2.09	0.47	0.99
5.5	...	0.86	0.67	0.09	0.45
6.5	...	1.37	0.25	0.09	0.09
7.5	...	0.26	0.08	...	0.09
8.5	...	0.34	0.08	...	...
9.5	...	0.26	...	...	...
10.5	...	0.09	...	...	...
	100.00	100.00	100.00	100.00	100.00
N[f]	1,417	1,164	1,196	1,072	1,107
S[g]	2.73×10^4	1.38×10^4	1.87×10^4	2.58×10^4	2.20×10^4
d_{ao}[h]	1.39	1.73	1.57	1.29	1.32
s[i]	0.71	1.37	1.06	0.68	0.83
	—Safflower Oil-Tween 40/Span 80 Emulsion—			—Safflower Oil-Pluronic F-68/—Span 80 Emulsion	
1.0	82.95	64.36	69.16	64.34	71.78
2.5	13.10	19.45	19.53	26.99	16.57
3.5	3.21	12.57	9.35	7.67	8.43
4.5	0.74	2.51	1.59	0.65	1.99
5.5	...	0.81	0.28	0.14	0.76
6.5	...	0.30	0.09	0.07	0.47
7.5	...	...	...	0.07	...
8.5	...	...	...	...	...
9.5	...	...	...	...	...
10.5	...	...	...	0.07	...
	100.00	100.00	100.00	100.00	100.00
N[f]	1,214	1,352	1,070	1,382	1,056
S[g]	2.66×10^4	1.90×10^4	2.12×10^4	2.09×10^4	1.92×10^4
d_{ao}[h]	1.33	1.75	1.60	1.64	1.59
s[i]	0.66	1.11	0.97	0.95	1.05

[a] Oil and Span in inner funnel. [b] Water and Tween in outer funnel. [c] Oil, Span, and Tween in inner funnel. [d] Water and Pluronic in outer funnel. [e] Frequency expressed as per cent of N. [f] N, Total counts. [g] S, Specific interfacial area. [h] d_{ao}, Arithmetic mean globule diameter. [i] s, Standard deviation. [j] cm.^{2}/ml. [k] Microns.

Effect of Exposure Time.—The effect of varying the insonation period of the emulsion in the Minisonic was studied. Prolonged exposure at certain frequencies will cause coarsening of the emulsion. Campbell and Long (7) and Alexander (5) reported this coagulation effect which results from collisions of dispersed particles. Higher frequencies increase the rate and energy of these collisions. The low operating frequency of the Minisonic (22 kc.) would not be expected to produce coarsening of the emulsion on prolonged exposure.

Emulsions Analyzed.—Five 25% o/w emulsions were prepared by the premix method and were irradiated in the Minisonic homogenizer for two and one-half, five, ten, fifteen, twenty, twenty-five, and thirty minutes at a constant temperature of 35°. The emulsions were photographed and analyzed as described. The emulsions used were: (*a*) mineral oil with 4% Tween 40/Span 80, (*b*) peanut oil with 6.25% acacia, (*c*) peanut oil with 4% Tween 40/Span 80, (*d*) peanut oil with 4% Pluronic F-68/Span 80, and (*e*) peanut oil with 1% sodium lauryl sulfate. Figure 1 shows the effect of the various exposure periods on the five emulsions.

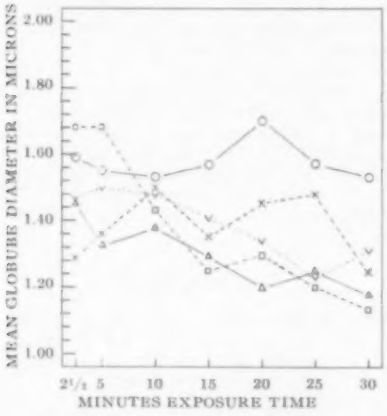

Fig. 1.—Effect on mean globule diameter of exposure to ultrasonic waves for various time intervals. Emulsions exposed: ○, mineral oil-Tween 40/Span 80; □, peanut oil-acacia; Δ, peanut oil-Pluronic F-68/Span 80; v, peanut oil-sodium lauryl sulfate; x, peanut oil-Tween 40/Span 80.

RESULTS

Table I shows that the method of size-frequency analysis used in this study gives a good degree of reproducibility or accuracy. Any error introduced by the analysis procedure is probably very small.

From Table II it can be seen that there was no appreciable difference in the three methods of forming the emulsions. The single-funnel premix method was at least equivalent to the two-funnel methods.

Figure 1 reveals that long insonation periods had no undesirable effects on the emulsions investigated. The low frequency of the Minisonic produced no coagulation in the emulsions. Three of the five emulsions showed decreasing mean globule diameters with increased exposure time.

Audouin and Levavasseur (8) reported that at 25 kc. o/w emulsions inverted to w/o emulsions if the exposure time exceeded fifteen minutes. If phase inversion is a characteristic of the low frequencies the emulsions of the Minisonic should have shown this phenomenon. The emulsions exposed for thirty minutes were checked by dilution and dye methods and were found to be universally oil-in-water.

SUMMARY

A simplified method of emulsion analysis has been perfected. This method has the advantages of being rapid, accurate, and of producing a permanent record. It is easier than previous photomicrographic techniques in that pictures do not have to be printed from the negatives. Measuring visually rather than mechanically with a calibrated rule materially speeds the analysis.

The Minisonic ultrasonic homogenizer was found to operate almost equally well regardless of the method used in making the emulsion. Prolonged insonation did not cause coagulation of the emulsions. In some instances there was a continual reduction in mean globule diameter; in other cases a plateau was evident from the very beginning of the homogenization.

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Emulsification With Ultrasonic Waves II*

Evaluation of Three Ultrasonic Generators and a Colloid Mill

By ROBERT E. SINGISER† and HAROLD M. BEAL

Three ultrasonic generators and a colloid mill were evaluated with 25 per cent mineral oil, peanut oil, and safflower oil emulsions, each made with four emulsifier systems. Emulsions were exposed in each apparatus for periods up to fifteen minutes. The best of these emulsions were analyzed after zero, fifteen, thirty, and sixty days storage. From the size-frequency analysis data specific interfacial area, mean globule diameter, and standard deviation were calculated. The Minisonic homogenizer produced emulsions distinctly superior to those made on any other apparatus.

EMULSIFICATION of vegetable and mineral oils with ultrasonic waves has progressed rapidly in the past decade and markedly so in the last several years. Prior to 1949 no extensive work was reported. Since that time significant contributions to this field have been made. Campbell and Long (1) and Audouin and Levavasseur (2, 3) experimented with emulsification of vegetable, mineral, and fish oils. The latter investigators found the ultrasonic frequency employed to be a preponderant factor in the orientation of the emulsion that formed. Beal and Skauen (4, 5) studied some of the factors influencing ultrasonic emulsification, and the effect of ultrasonic waves on various emulsifying agents.

Although many claims have been made for ultrasonic apparatus as homogenizers no comparative evaluation has been attempted. This study was undertaken to provide a measure of comparison between various ultrasonic generators and between the ultrasonic homogenizers and a conventional homogenizer. As a secondary observation the relative efficiency of the various emulsifying agents and the ease of emulsification of different oils was noted.

APPARATUS

Four apparatus were studied; they were assigned codes as follows:

Code 1.—The Eppenbach Colloid mill, model QV-6-1, was used to represent conventional emulsifying equipment with which to compare the ultrasonic generators. The rotor-stator clearance was adjusted so that flow rate through the recirculation pipe was 700–900 ml./min. The water-cooled jacket was utilized throughout.

Code 2.—General Electric model G-3 piezoelectric-type ultrasonic generator with a quartz crystal operating at 300 kc. and a plate current of 175 ma. Irradiation of the sample was done in a 2-ounce polyethylene bottle positioned to give maximum activity in the water-cooled chamber. Sample size was limited to 20 ml.

Code 3.—Branson Ultrasonic Corp. Sonogen generator, model AP-25-B, driving at 39.1 kc. barium titanate tank-type transducer, model T-52. This piezoelectric-type generator was tuned to operate between 80–100 ma. One hundred-milliliter samples in 8-ounce polyethylene bottles were partially immersed in the water-filled tank above the transducer. Very little activity was produced and it was necessary to provide slow speed stirring to keep the emulsion components reasonably well dispersed in order that emulsification would occur.

Code 4.—Minisonic Mark 2 hydrodynamic-type ultrasonic generator manufactured by Sonic Engineering Corp. The single funnel method for using this generator (6) was employed in this study. One-liter quantities were recycled in this water-cooled apparatus.

EXPERIMENTAL

Emulsion Components.—Three oils were selected for use in this investigation: heavy mineral oil U. S. P., peanut oil U. S. P., and safflower oil, edible (Pacific Vegetable Oil Corp.).

Four emulsifier systems were used in this study: (*a*) acacia in the same oil-gum ratio as the official acacia emulsion; (*b*) Tween 40[1]/Span 80[1]; (*c*) Pluronic F-68[2]/Span 80; and (*d*) sodium lauryl sulfate.

Distilled water containing 0.2% of the methyl and 0.02% of the propyl esters of *p*-hydroxybenzoic acid was used in the external phase of all emulsions. The safflower oil contained 0.01% propyl gallate and 0.01% citric acid as antioxidants. These antioxidants in the same concentration were also added to the aqueous phase of all safflower oil emulsions.

Formulas of Emulsions.—Table I shows the twelve basic 25% emulsions used in this study. Each of the three oils was prepared with each of the four emulsifier systems. The oils and emulsifier systems were coded so that easier identification of each combination was possible: M—mineral oil, P—peanut oil, S—safflower oil, A—acacia, B—Tween 40/Span 80, C—Pluronic F-68/Span 80, and D—sodium lauryl sulfate. Where possible the HLB system (7) of classifying and selecting emulsion com-

* Received August 21, 1959, from Abbott Laboratories, Scientific Division, North Chicago, Ill.

This paper is based on a dissertation submitted by Robert E. Singiser to the Graduate School of the University of Connecticut in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

† Fellow, American Foundation for Pharmaceutical Education.

[1] Product of Atlas Powder Co., Wilmington, Del.

[2] Product of Wyandotte Chemical Co., Midland, Mich.

TABLE I.—25% v/v O/W EMULSIONS STUDIED

Code	Oil	HLB	Acacia	Tween 40,[a] % (HLB 15.6)	Span 80,[a] % (HLB 4.3)	Pluronic F-68,[a] % (HLB 16)	Sodium Lauryl Sulfate, %	Distilled Water,[b] % q. s.
M-A	Mineral oil	12.0	6.25	...	...	...	...	100
M-B	Mineral oil		...	2.72	1.28	...	...	100
M-C	Mineral oil		...	...	1.36	2.64	...	100
M-D	Mineral oil		...	...	...	...	1.00	100
P-A	Peanut oil	8.0	6.25	...	...	...	...	100
P-B	Peanut oil		...	1.32	2.68	...	...	100
P-C	Peanut oil		...	...	2.72	1.28	...	100
P-D	Peanut oil		...	...	...	...	1.00	100
S-A	Safflower oil	11.5	6.25	...	...	...	...	100[c]
S-B	Safflower oil		...	2.52	1.48	...	...	100[c]
S-C	Safflower oil		...	...	1.52	2.48	...	100[c]
S-D	Safflower oil		...	...	...	...	1.00	100[c]

[a] Total mixed emulsifier concentration 4%. [b] Preserved with 0.2% methyl and 0.02% propyl esters of *p*-hydroxybenzoic acid. [c] Antioxidants added: 0.01% propyl gallate and 0.01% citric acid.

ponents was used in formulating the emulsions. HLB values for Pluronic F-68 and safflower oil were experimentally determined, the other HLB values were obtained from the literature (7, 8). Table I lists these values.

Premixes of each of the twelve basic emulsions were prepared by shaking in a gallon bottle. This permitted uniform aliquots to be withdrawn and emulsified in the various apparatus. The orientation of each of the emulsions was confirmed to be o/w by the addition of dyes and by dilution techniques.

Evaluation.—Each of the twelve emulsions was insonated in each of the four apparatus for two and one-half, five, ten, and fifteen minutes. Each emulsion thus formed was evaluated by the photomicrographic size-frequency method previously described (6). A minimum of 1,000 globules was measured for each sample. The best emulsion from the four insonation periods was then stored at room temperature and analyzed again at fifteen, thirty, and sixty days.

A 50% propylene glycol solution was used as the diluting fluid for all emulsions except those made with acacia. The acacia-stabilized emulsions broke when diluted with propylene glycol so a 12.5% acacia solution was used. Both fluids effectively prevented Brownian movement and have been previously reported as satisfactory diluents (4, 9, 10).

Code.—The code identifies the emulsion as to the oil used (M-P-S), the emulsifier system (A-B-C-D), and the homogenizer (1-2-3-4). A typical code reads M-A-4, indicating a mineral oil emulsion (M) with acacia (A) as the emulsifier, the emulsion being made on the Minisonic (code 4) homogenizer.

Parameters.—The specific interfacial area and the arithmetic mean diameter with standard deviation were calculated from the analysis data. The specific interfacial area (S) was calculated following the method of Mullins and Becker (8). While this value is often reported alone it is difficult to visualize and gives very little indication of the type of distribution it represents. The inclusion or exclusion of one globule in the smaller class intervals has little effect on the specific interfacial area while in the larger class intervals this one globule changes the value considerably. The mean globule diameter (d_{as}) is not subject to such weighted influence of one globule. The mean globule diameter with standard deviation (s) gives a good indication of the type of distribution in a sample. The standard deviation has been used by Jellinek (11) who described it as the inhomogeneity factor. A large value indicates much variance in the distribution and a small value indicates high uniformity.

Complete data on all of the emulsions analyzed cannot be given here. The mean globule diameter was selected as representative of the results obtained. These data for the stored emulsions are graphed as follows: Figures 1–4 illustrate the mineral oil emulsions, Figs. 5–8 the peanut oil emulsions, and Figs. 9–12 the safflower oil emulsions.

DISCUSSION

Due to crystal fracture on the General Electric unit some emulsions could not be made on it. The unit's small capacity precludes any commercial use of this generator as a homogenizer. Beal and Skauen (4, 5) have done extensive work with this unit.

Where graphs show only a single "0 days" entry for certain emulsions, free oil was found at the fifteen-day observation. No further analysis was made when this gross instability was observed.

Apparatus.—Figures 1–12 indicate that the Minisonic homogenizer produces emulsions superior to those produced on the other apparatus. Generally, the emulsions made on the Minisonic had a smaller mean globule diameter (or larger specific interfacial area) than any others. These emulsions also had greater uniformity. With the exception of the mineral oil emulsions, the Minisonic-made emulsions showed very few particles larger than 5.5 μ. This degree of uniformity was not found in any other apparatus. The Eppenbach mill, the General Electric, and Sonogen generators were approximately equivalent in the degree of dispersion produced.

Of the apparatus evaluated only the Eppenbach mill and the Minisonic generator are adaptable for production use. It would be expected that the fifteen-minute exposure samples from the Eppenbach mill would be best. The rotor-stator arrangement simply provides shear for disrupting the oil phase. In three instances (M-A, M-D, S-A) samples other than the fifteen-minute exposure samples were found to be best. Of all the units the Eppen-

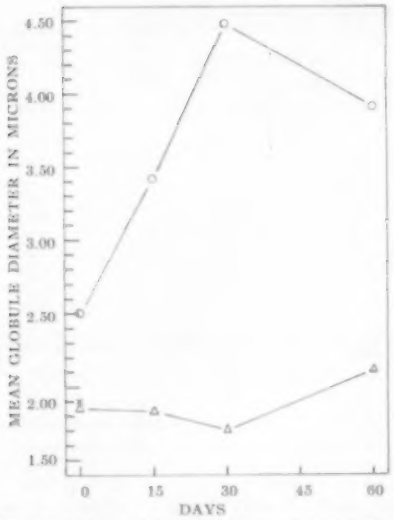

Fig. 1.—Mean globule diameters of stored mineral oil-acacia emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; △, Minisonic ultrasonic generator; x, Sonogen ultrasonic generator.

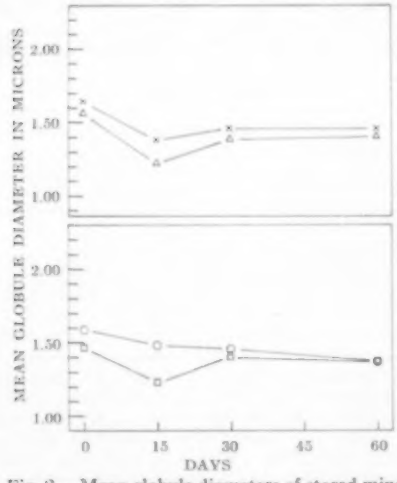

Fig. 2.—Mean globule diameters of stored mineral oil-Tween 40/Span 80 emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

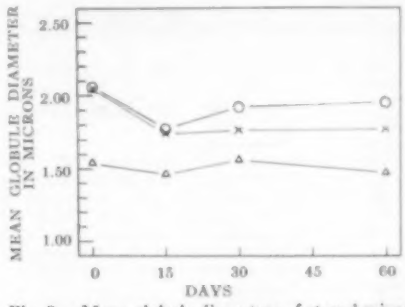

Fig. 3.—Mean globule diameters of stored mineral oil-Pluronic F-68/Span 80 emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

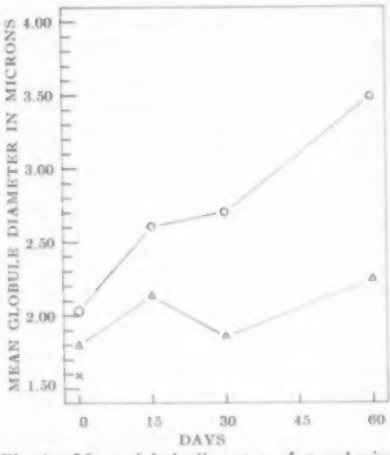

Fig. 4.—Mean globule diameters of stored mineral oil-sodium lauryl sulfate emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

bach mill had the greatest tendency to incorporate air into the emulsion. It is recognized that air entrapment in emulsions may accelerate their breakdown (12). This may be the reason for the inferior long-exposure samples.

Table II summarizes the mean globule diameters and average specific interfacial area produced by the different apparatus. These figures are the average of the four stability storage results. Since few of the emulsions underwent any marked deterioration during the sixty days storage this average value is useful for comparisons, but it has no value beyond that. This lack of change in the emulsions undoubtedly parallels the findings of Harkins and Beeman (13) who noted that when sufficient emulsifying agents were used to complete a monomolecular layer about the oil droplets, the emulsion could remain appreciably unchanged for as long as one year. King and Mukherjee (14) found vast stability differences by changing emulsifier concentration.

The Eppenbach mill had an aggregate average particle diameter of 2.36 μ, the Minisonic generator produced globules averaging 1.48 μ. These average values represent measurements on approximately 55,000 globules.

The 300 kc. frequency of the General Electric

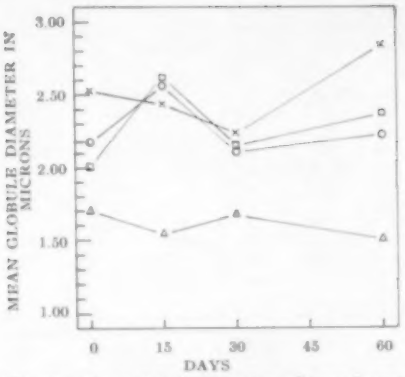

Fig. 5.—Mean globule diameters of stored peanut oil-acacia emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

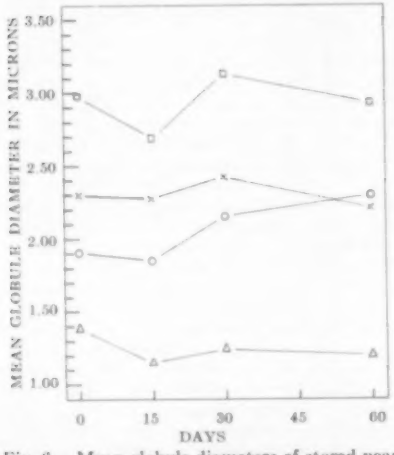

Fig. 6.—Mean globule diameters of stored peanut oil-Tween 40/Span 80 emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

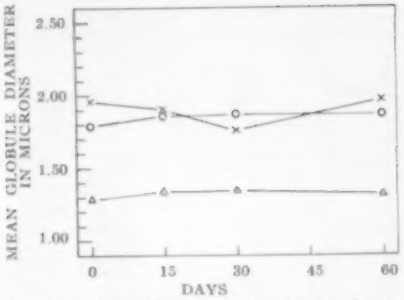

Fig. 7.—Mean globule diameters of stored peanut oil-Pluronic F-68/Span 80 emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

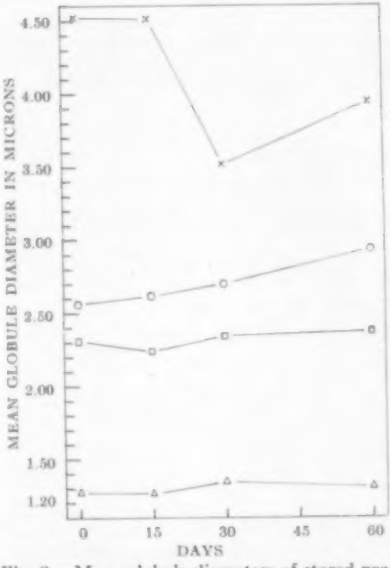

Fig. 8.—Mean globule diameters of stored peanut oil-sodium lauryl sulfate emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

unit has a marked tendency to cause coagulation of the emulsion on prolonged exposure. In all cases the ten-minute exposure samples were better than the fifteen minute ones. Using the same generator Beal and Skauen (5) found a gradual improvement in their emulsions up to thirty minutes. In this study a polyethylene bottle was used as the exposure chamber. Polyethylene has poor heat transfer qualities. Even though the bottle was immersed in a cooling bath, temperatures of 55–60° developed in the emulsion on fifteen minutes insonation. Bondy and Sollner (15), Audouin and Levavasseur (2), and others (16) have noted that elevation of temperature does not favor emulsification, but actually favors coagulation.

The only emulsions to show separation of free oil were those prepared in the Sonogen generator. The Sonogen is not well suited for the production of emulsions. The ultrasonic waves produce very little agitation in the exposure tank, and as a result some type of stirring must be provided to keep the emulsion components in intimate contact. In this study minimal stirring was employed. Even so, it is questionable how much of the emulsification is attributable to stirring and how much is actually due to ultrasonic cavitation. In addition, there is no way to maintain a constant temperature with this apparatus, which is designed mostly as a cleaning tool.

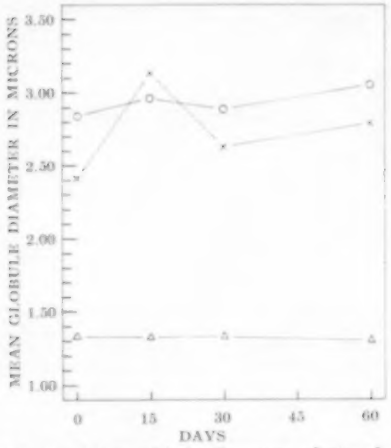

Fig. 9.—Mean globule diameters of stored safflower oil-acacia emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

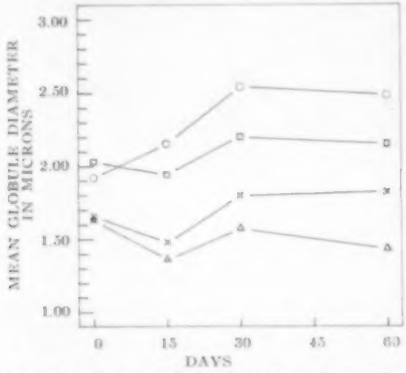

Fig. 11.—Mean globule diameters of stored safflower oil-Pluronic F-68/Span 80 emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

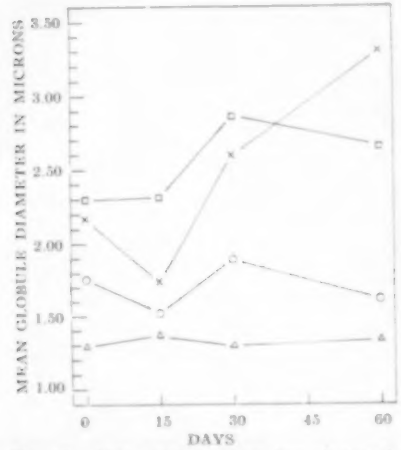

Fig. 10.—Mean globule diameters of stored safflower oil-Tween 40/Span 80 emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

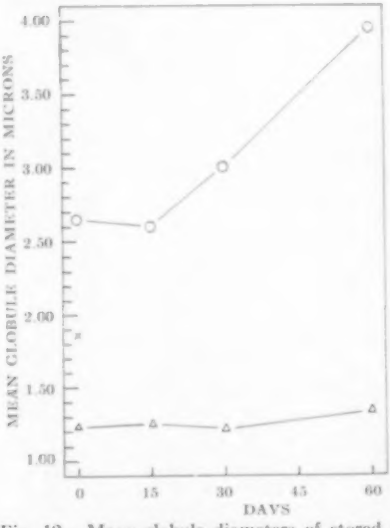

Fig. 12.—Mean globule diameters of stored safflower oil-sodium lauryl sulfate emulsions. Emulsifying apparatus: ○, Eppenbach colloid mill; x, Sonogen ultrasonic generator; △, Minisonic ultrasonic generator.

Emulsions.—Sodium lauryl sulfate-stabilized emulsions showed the greatest tendency for instability, especially when prepared in the Eppenbach colloid mill (M-D-1, M-D-4, P-D-1, S-D-1). These emulsions have a very low viscosity which more than likely contributes to their instability. King and Mukherjee (14) found a high viscosity essential to good stability.

Table II shows that the nonionic emulsifier pairs, Tween 40/Span 80 (series B) and Pluronic F-68/Span 80 (series C), were generally the best of the agents used. Neither of these emulsion systems showed oil separation. Siragusa, *et al.* (17), found a Tween 80/Span 20 emulsifier combination better for a 12.5% mineral oil emulsion than acacia, both made in the Eppenbach mill. King and Mukherjee noted that the efficiency of an emulsifying agent is a very specific property. Mixed emulsifiers should give better emulsions than the single agents since it is possible to match the emulsifier HLB with the HLB required by the oil.

According to the work of Chun, *et al.* (18), the HLB value of acacia is 8.0. Since the HLB requirement of peanut oil is also 8.0 for an o/w emulsion

TABLE II.—SUMMARY OF DATA ON EMULSIONS[a]

Emulsion	Eppenbach			General Electric			Sonogen			Minisonic		
	d_{av}[b]	s[c]	S[d]	d_{av}	s	S	d_{av}	s	S	d_{av}	s	S
M-A	3.61	3.12	0.74	[e]	[e]	[e]	[f]	[f]	[f]	1.88	1.30	1.58
M-B	1.48	1.13	1.43	1.37	0.88	1.95	1.49	1.04	1.66	1.40	1.00	1.64
M-C	1.93	1.04	2.03	[e]	[e]	[e]	1.84	0.98	2.13	1.52	0.84	2.41
M-D	2.68	2.74	0.75	[e]	[e]	[e]	[f]	[f]	[f]	2.02	1.69	1.20
P-A	2.26	1.80	1.12	2.28	1.80	1.06	2.48	2.35	0.88	1.61	0.83	2.46
P-B	2.05	1.82	0.99	2.92	2.25	0.94	2.30	1.50	1.35	1.26	0.60	3.11
P-C	1.85	1.49	1.26	[e]	[e]	[e]	1.90	1.78	1.03	1.33	0.76	2.44
P-D	2.71	2.47	0.84	2.31	2.14	0.91	4.06	3.12	0.72	1.30	0.61	3.09
S-A	2.94	2.56	0.83	[e]	[e]	[e]	2.75	2.58	0.82	1.33	0.66	2.91
S-B	1.70	1.40	1.24	2.52	1.77	1.13	2.45	2.60	0.78	1.33	0.70	2.68
S-C	2.27	2.01	1.02	2.08	1.68	1.21	1.69	1.34	1.41	1.51	0.84	2.40
S-D	3.06	2.54	0.88	[e]	[e]	[e]	[f]	[f]	[f]	1.27	0.59	3.15

[a] Average of zero-, fifteen-, thirty-, and sixty-day determinations.
[b] d_{av}, Arithmetic mean globule diameter, μ.
[c] s, Standard deviation: $s = \sqrt{\frac{n(\Sigma fx^2) - (\Sigma fx)^2}{n(n-1)}}$, μ.
[d] S, Specific interfacial area, $\times 10^4$ cm.^{2}/ml.
[e] Generator failure, no emulsion prepared.
[f] Oil separation at fifteen days.

it would be anticipated that the peanut oil-acacia (P-A) emulsions would be superior to mineral oil-acacia (M-A) or safflower oil-acacia (S-A) emulsions. Table II shows that this is generally true. The mean globule size of all P-A emulsions is 2.13 μ, 2.67 μ for M-A emulsions, and 2.32 μ for S-A emulsions. The acacia emulsions were uniformly coarser than the Tween 40/Span 80 or Pluronic F-68/Span 80 emulsions. King and Mukherjee (14) found emulsions stabilized by hydrophilic colloids to be generally coarse, but usually stable, at least in part due to their high viscosity.

From the data it appears that all three of the oils have approximately the same ease of emulsification when handled as they were in this investigation.

In diluting the emulsions for photomicrography a preliminary indication of the better emulsions was observed. Generally dilutions of 1:100 to 1:200 produced fields suitable for measurement. In emulsions prepared in the Minisonic it was necessary to dilute to 1:450 to get few enough particles to analyze satisfactorily. Even at this dilution there were many more globules in the field than found with the other emulsions. Emulsions other than the Minisonic ones broke when such extreme dilutions were made. The counting method used by Beal and Skauen (4, 5) where exact dilutions are made would undoubtedly have shown the Minisonic emulsions to be even more superior than the evaluation methods of this study showed.

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Book Notices

Einführung in die Pharmakopsychologie. By HERBERT LIPPERT. Band 4 der Enzyklopädie der Psychologie in Einzeldarstellungen. Verlag Hans Huber, Bern and Stuttgart, 1959. Intercontinetal Medical Book Corp., 381 Fourth Ave., New York 16, N. Y. 254 pp. 15 × 23 cm. Price Fr./DM 32.

This book (in German) is intended as an introductory textbook in pharmacopsychology. The final chapter covers the relation of chemical structure and psychic action of barbiturates, phenylaminoethane derivatives, opium alkaloids, and synthetics, cocaine-atropine group, alcohol-ether group, and ataractic-tranquilizers.

Recent Advances in Neuro-Physiological Research. Edited by D. EWEN CAMERON and MILTON GREENBLATT. American Psychiatric Association, 1700 Eighteenth St., N. W., Washington 9, D. C., 1959. 136 pp. 15 × 23 cm. Paperbound.

This is a compilation of nine reports on recent research in this field; covering communication, memory disturbance, electrophysiology, animal behavior, and a biological basis for psychopathology. Discussions of the papers are included, but there is no index.

The United States Pharmacopeia. 16th rev. Published by the U. S. Pharmacopeial Convention, Inc., 1960. Distributed by Mack Publishing Co., Easton, Pa. xliv + 1148 pp. 15 × 23 cm. Price: $10 domestic, $10.50 foreign.

The U. S. P. revision committees have maintained such a high degree of excellence for so long that when a new revision of the compendium appears we are inclined to accept it as something automatically produced. U. S. P. XVI unquestionably reflects the competent medical and pharmaceutical efforts of a dedicated group of experts in these fields. The 908 monographs in U. S. P. XVI include 225 new admissions, which represent every new class of drugs introduced since 1955. These include the oral antidiabetics, orally effective diuretics, antihypertensives, and anticarcinogenic agents. In addition there are listed 81 titles of "U. S. P. XVI interim admissions." Monographs for these articles, which have been approved for admission by the U. S. P. Committee on Scope, are still being developed and should appear in Interim Revision Announcements or in Supplements to U. S. P. XVI. Progressive analytical studies are reflected in the applications of new analytical techniques such as infrared spectrophotometry, chromatography, radioisotope tracers, etc., to drugs in retained monographs as well as in new monographs. The increase in spectrophotometric procedures for identification tests and assays is reflected in the addition of 78 new U. S. P. Reference Standards, most of them being required for spectrophotometric methods.

The style and format of the monograph section of the book is similar to that in U. S. P. XV but the section headed "General Tests, Processes and Apparatus" is set in space-saving two-column format. This section covers 272 pages and has its separate table of contents. On behalf of the U. S. P. revision committee and those who assisted in the preparation of U. S. P. XVI, chairman Lloyd C. Miller and his executive assistant Adley B. Nichols should receive our commendation. The new official compendia, U. S. P. XVI and N. F. XI, the technical revision of which is attributable mainly to pharmacy members of the revision committees, are splendid proof that pharmacy can set its standards high in the professional aspects of its practice.

Optical Rotatory Dispersion. By CARL DJERASSI. McGraw Hill Book Co., 330 West 42nd St., New York 36, N. Y. 1960. xiii + 293 pp. 15 × 23 cm.

This book deals with rotatory dispersion as a physical tool used to solve structural, stereochemical, and conformational problems in organic chemistry and biochemistry. It considers mainly the advances made since 1955 with particular emphasis on steroids, terpenes, amino acids, and proteins and peptides. The increased interest and experimental work in the field has been greatly stimulated by the acquisition of spectropolarimeters by many laboratories. Three of the 17 chapters have been written by experts in their fields of instrumentation and application of optical rotatory dispersion. Appendixes include a bibliography of publications, an explanation of the nomenclature, and stereochemical conventions for steroids and triterpenoids. Author and subject indexes are given.

Stress and Cellular Function. By H. LABORIT, *et al.* J. B. Lippincott Co., Medical Dept., East Washington Square, Philadelphia 5, Pa., 1959. xi + 255 pp. 15.5 × 23 cm. Price $7.50.

This book, intended for the clinician, is a translation of the original French edition which had the title "Resuscitation: Physio-biological Bases and General Principles." The author attempts to outline three basic concepts from which he proposes to develop a series. He states: "Knowledge of systemic reaction to injury under its diversified aspects, will lead to a particular understanding of its management as well as of *resuscitation*." Resuscitation is the term used to indicate the restoration to normal values found in the healthy body.

Technique et Pratique de la Correction Prothétique des Surdités. By JACQUES DEHAUSSY. Masson et Cie., Éditeurs, 120, boulevard Saint-Germain, Paris 6e, France, 1960. 265 pp. 17 × 25 cm. Paperbound. Price NF. 30,000.

This book (in French) includes methods of testing hearing ability, diagnosing abnormalities, and describes corrective procedures. It is directed to the specialist in this branch of medical practice.

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